Ventilator Induced Lung Injury

Pathophysiology of mechanotransduction and therapeutic strategies

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Pathophysiology of mechanotransduction and therapeutic strategies

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"But so that life may in some measure be restored to the animal, you must attempt an opening in the trunk of the trachea and pass into it a tube of rush or reed, and you must blow into this so that the lung may expand and the animal draw breath after a fashion; for at a light breath the lung in this living animal will swell to the size of the cavity of the thorax, and the heart take strength afresh and exhibit a great variety of motions".

This translation from the original Latin text is the first account of mechanical ventilation. The experiment was performed on a pregnant sow by Andreas Vesalius and reported in the last chapter of his famed anatomical treatise "De humani coporis fabrica" published in 1543.

Ter nagedachtenis aan mijn vader
Voor mijn moeder

CONTENTS

Chapter 1

General Introduction

Adapted from

Cytokines and Biotrauma in Ventilator Induced Lung Injury:
a critical review of the literature .

Michiel Vaneker, Feico J.J. Halbertsma, Gert Jan Scheffer, Johannes G. van der Hoeven.

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HISTORICAL OVERVIEW

In 1745, John Fothergill presented the first interesting case report addressing the issue of lung injury induced by artificial respiration.[1] The case involved an apneic, pulseless individual who had collapsed due to noxious fumes from a coal pit. As described by Fothergill, a physician by the name of Tossack applied his mouth close to the patient, and by blowing strongly, pulling the nostrils at the same time, raised his chest fully by his breath. Immediately six or seven very quick beats of the heart were felt, the thorax movement continued and a pulse was soon felt in the arteries. Within one hour, the patient began to come round and walked home within four hours and after a couple of days returned to work. Fothergill suggested that mouth-to-mouth resuscitation may be better than using a mechanical method of insufflating the lungs with air using a pair of bellows because "the lungs of one man may bear, without injury, as great a force as those of another man can exert; which by the bellows cannot always be determined". Thus, Fothergill had an early understanding of the concept of ventilator induced lung injury (VILI).

The age of widespread mechanical ventilation began after the introduction of the whole body negative pressure device, tank respirator (figure 1). The device was a large, airtight metal cylinder that enclosed the patient, exposing only the head and neck. Negative pressure was generated with an electric pump, causing the patient's chest to rise. This device was particularly effective as a noninvasive form of mechanical ventilation for patients with normal airways and it was extensively used in the mid-1950s during the poliomyelitis epidemic. However after the introduction of repeated blood gas analysis it was found that negative pressure ventilation failed to provide adequate ventilation. Positive pressure ventilation with the use of an artificial airway replaced the negative pressure support technology. Soon different modes of positive

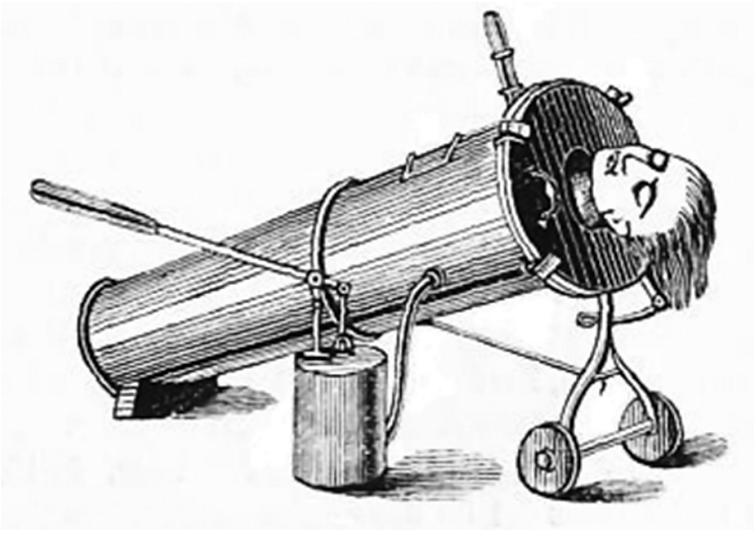

Figure 1: Iron lung

pressure ventilation were introduced and the modern era of respiratory support was born including controlled mechanical ventilation and assisted ventilation.

In 1974, Webb en Tierney demonstrated that mechanical ventilation with high peak airway pressures resulted in the development of a pressure gradient between an alveolus and the adjacent bronchovascular sheath, causing lung edema, alveolar disruption and air leakage.[2] This has been termed "barotrauma". The clinical and radiological manifestations of "barotrauma" include pneumothorax, pneumomediastinum, pneumoperitoneum and subcutaneous emphysema.[3;4] Soon studies investigated the physical forces and challenged the concept of "barotrauma". For example, mechanical ventilation of rabbits with high peak inspiratory pressures (45 cm H_2O) resulted in severe pulmonary edema and gross lung injury. In contrast, in rabbits encased in plaster casts, ventilation with similar airway pressures resulted in less lung injury.[5] Thus alveolar overdistension and not the end-inspiratory pressure per se was hypothesized as the main determinant.[6] Accordingly, the term "volutrauma" was introduced.

Subsequent studies showed that cyclic opening and collapsing of alveoli, even at low inspiratory pressures and low inspiratory volumes, increased stretch- and shear forces resulting in lung injury and surfactant dysfunction.[7;8] This "atelectrauma" could be attenuated by increasing Positive End-Expiratory Pressure (PEEP) and outweighed the concomitant increase in inspiratory pressure.[2;9]

Although little doubt exists that mechanical ventilation (especially at high volume) can physically disrupt the lung, recent studies have shown that mechanical ventilation also results in more subtle morphological and functional changes and can excite an inflammatory response within the lung, a mechanism called "biotrauma".[10-12] Figure 2 shows a historical overview of mechanical ventilation-induced lung injury.

VENTILATOR INDUCED LUNG INJURY

Clinical aspects:

Mechanical ventilation is one of the cornerstones of treatment in the intensive care unit. Despite its lifesaving effects, mechanical ventilation may lead to serious damage in both previously healthy and diseased lungs.[2;6;10;11;13;14] In its most severe form VILI can result in Acute Respiratory Distress Syndrome (ARDS). The diagnostic criteria for ARDS include: acute onset; bilateral chest infiltrates; pulmonary artery occlusion pressure of 18 mm Hg or less or no evidence of left atrial hypertension; impaired oxygenation regardless of the level of PEEP with a ratio of partial pressure of oxygen in arterial blood (PaO_2) to fractional intake of oxygen (FiO_2) of less than 200.[15] In short, the syndrome is characterized by leaky, stiff lungs and severe hypoxemia, associated with inflammatory alveolar fluids.[15] The reported mortality of ARDS is still 30 to 50%.[15-19] Interestingly, most patients with ARDS do not die from respiratory failure but from multiple organ dysfunction syndrome (MODS).[20] Why would a patient having a disease that primarily

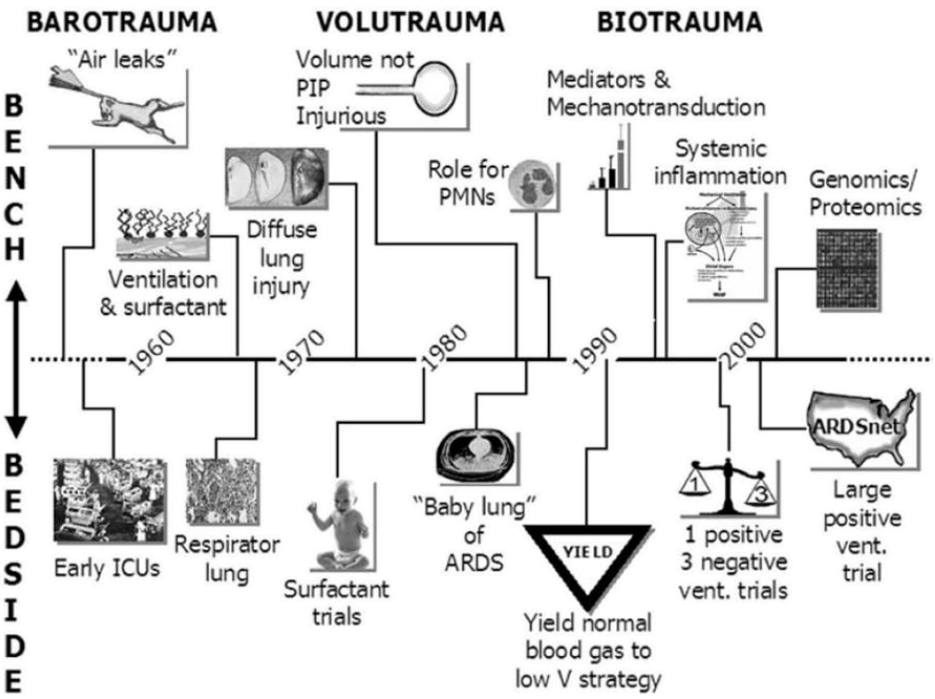

Figure 2: Historical overview of mechanical ventilation-induced lung injury.

affects the lungs eventually die from distal organ failure? One challenging hypothesis is that the inflammatory response originating from the lungs "spreads out" and triggers a systemic inflammatory response. Indeed, several studies have found that mechanical ventilation is associated with a systemic inflammatory response. Moreover, ventilator settings, especially tidal volume, have been shown to affect the severity of the inflammatory response.[13;21-23] In patients with ARDS the highest cytokine concentrations are found downstream from the lung,[24] supporting the notion that biotrauma is not only confined to the lungs.[3;3;10-12;21;25;25;25-31] Although studies have shown an association between pulmonary and systemic inflammatory mediators and outcome in ARDS patients,[13] a causal relationship has not been proven. Nevertheless, a more in depth insight into the inflammatory pathways associated with mechanical ventilation may prove valuable in developing therapeutic strategies to limit inflammation in mechanically ventilated patients.

Cytokines:

Cytokines are low-molecular weight soluble proteins that transmit signals between cells involved in the inflammatory response.[32] In the lung, cytokines originate from bronchial, bronchiolar and alveolar epithelial cells,[33;34] alveolar macrophages and neutrophils.[35] It is now commonly accepted that increased production of cytokines in the lung plays a key role in VILI.[36]

Several mechanisms have been proposed to initiate the enhanced generation of cytokines with mechanical ventilation including alterations in cytoskeletal structure without ultrastructural damage (mechanotransduction),[37;38] stress failure of the alveolar barrier (decompartimentalization),[39] stress failure of the plasma membrane (necrosis),[40] and effects on the pulmonary vasculature independent of stretch or rupture.[10]

To examine the mechanisms of cytokine generation during mechanical ventilation, *in-vitro*, *ex-vivo* and *in-vivo* models, have been studied.[36] Upon stretch, alveolar cells produce inflammatory mediators such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-8, IL-10,[34;35;41-45] *in vitro,*[34;35;44;46]. Ventilation with large tidal volume results in a similar inflammatory response both *ex-vivo,*[47] and *in-vivo*.[12;39;48;49]

Clinical studies have shown that the inflammatory response in the lung depends on ventilator settings. Transiently increasing tidal volume from 5 to 12 ml/kg and reducing PEEP from 15 to 5 cmH_2O in acute lung injury patients, increases levels of IL-6, IL-10 and TNF-α levels in both broncho-alveolar lavage (BAL) fluid and plasma within one hour.[13;50] These findings are consistent with the results of Ranieri et al., reporting lower cytokine levels in BAL fluid of patients ventilated with low tidal volume,[21] and those of the ARDS network trial that found lower plasma IL-6 levels in the low tidal volume group.[13]

Despite the fact that low tidal volume ventilation ameliorates the inflammatory response, subjects are still in a "pro-inflammatory state" during mechanical ventilation. It should be noted that we do not yet know the ventilatory strategy that is the most effective in limiting inflammation.

Leukocytes:

The activation and attraction of leukocytes to the lungs is an important feature in VILI.[51-53] Under normal conditions leukocytes must undergo a considerable change in shape to squeeze through the small pulmonary capillaries, which increases transit time, creating a "physiological sink" of leukocytes in the lung.[54] Therefore a large pool of leukocytes, which is available at all times, exists in the lung. Leukocytes are predominantly activated and attracted to the lungs by chemokines.[53] Keratinocyte-derived chemokine (KC; IL-8 homologue) is the most potent leukocyte attractant.[55] Experimental leukocyte depletion is associated with dampened KC response in the lungs of animals.[51;52] Moreover, leukocyte depletion significantly reduces VILI as indicated by improved gas exchange and reduced pulmonary protein leak compared to non depleted animals. [51] Interestingly, alveolar recruitment of leukocytes by instilling a chemo attractant does not result in lung injury,[56] indicating that other mediators, possibly cytokines, are necessary to activate the leukocytes.

Role for Toll-like receptors:

The Toll-like receptors owe their name to the closely related receptor called Toll, first identified in Drosophila in 1988 during a screen for polarity in early embryogenesis.[57] Subsequently, it was shown that Toll gene mutant flies are highly susceptible to fungal infection, demonstrating that Toll is an important receptor in the innate immune system for detecting the invasion of microorganisms.[58] Since 1997, mammalian homologues of the Toll receptor were identified and designated as Toll-like receptors (TLRs). TLRs in mammals have been a major focus for research in immunology. To date, 11 human TLRs and 13 mouse TLRs have been identified.[59] TLRs appear to act as primary sensors by virtue of their capacity to recognize various microbes and initiate an immune response.[60-63] TLR expression is not restricted to immunological cells, but has also been found in lung tissue.

Interestingly, more recent data demonstrated that TLRs not only recognize microbial products, but also endogenous ligands. These ligands have been called 'danger signals'.[64;65]

Most research on lung injury has focused on TLR2 and TLR4. Activation of TLR4 with lipopolysaccharide induces a pro-inflammatory response.[66;67] Lipopolysaccharide is a component of the outer membrane of Gram-negative bacteria. After binding to TLR4,[68] it induces an intense inflammatory response associated with lung injury.[69-71]

Lung injury induced by hemorrhage,[72-74] ischaemic-reperfusion,[75] contusion,[76] hyperoxia or administration of bleomycin,[77] results in an inflammatory response that is also mediated via TLR4 and/or TLR2 supporting a role for these receptors in the development of non-infectious lung-injury. Endogenous ligands for TLR4 and TLR2 have now been identified and include heat shock protein 60,[78] fibronectin,[79] heparan sulfate proteoglycan,[80] biglycan,[81] hyaluronan[82] and the myeloid-related proteins 8 and 14.[83]

Downstream signaling of TLRs is complex (figure 3). Briefly, TLR signaling is initiated by the Toll/interleukin-1 receptor (TIR domain),[84] and mediated through a TIR domain containing adaptor protein: myeloid differentiation factor 88 (MyD88).[85] MyD88 is a critical adaptor molecule used by all TLRs.[86] In TLR4 an alternative, MyD88 independent pathway, was also identified. This pathway is mediated through a TIR domain containing adaptor called TRIF (Toll/interleukin-1 receptor domain-containing adapter inducing interferon-β). In TLR4 signaling TRIF as well as MyD88 activates nuclear factor-κB (NF-κB),[59] a DNA binding protein that plays a central role as a common messenger in cytokine regulation and inflammation. NF-κB activation is a critical step in the transcription of genes necessary to perpetuate the innate immune response, a process that starts within minutes after commencing mechanical ventilation.[87] In an experimental model the inflammatory response following high tidal volume ventilation was mediated by NF-κB,[47] and blockage of NF-κB decreased VILI.[11;35;47;52;88] As TLR4 and 2 have been shown to mediate non-infectious lung injury,[77] they are potential targets for the treatment of VILI, although no studies have proven this concept so far.

MODULATING THE INFLAMMATORY RESPONSE FOLLOWING MECHANICAL VENTILATION

Protective ventilation:

Mechanical ventilation-induced inflammation is associated with lung injury,[13] and peripheral organ dysfunction.[27] Furthermore, persistent cytokine elevation is associated with a poor outcome in patients with ARDS.[89;90] Therefore strategies to reduce mechanical ventilation-induced inflammation may be of clinical relevance. Low tidal volume mechanical ventilation was designed to protect the lungs from excessive stretch. This so called "lung protective mechanical ventilation" attenuates inflammation in patients with acute lung injury.[21;22] Furthermore the ARDS network study in 2000,[13] showed improved survival in these patients after treatment with low tidal volume mechanical ventilation. Despite these improvements no definitive treatment for ARDS has been found.

Hypercapnia:

Permissive hypercapnia (deliberate induction of alveolar hypoventilation and acceptance of hypercapnia) is a therapeutic approach designed to maintain oxygenation while preventing the risks caused by excessive airway pressures. Severe hypercapnic acidosis is usually well tolerated,[91] and one clinical study suggest a protective effect on various organs, including the lung.[92;93] A retrospective multi-variate logistic regression analysis of the ARDS network trial suggests that hypercapnic acidosis may be protective in ARDS patients ventilated with high tidal volumes.[94] An increase in carbon dioxide may have a direct modulating effect on the inflammatory response. Therefore hypercapnic acidosis is a strategy worth testing in clinical trials that aim to reduce VILI.

Isoflurane:

Isoflurane is widely used as a general inhalation anesthetic. It has profound protective immunological effects on the heart,[95] the brain,[96] and the kidneys.[97] In lung tissue, isoflurane was found to inhibit cytokine production in an in vitro model stimulating alveolar epithelial type II cells.[98;99] In vivo models also show isoflurane to reduce inflammation in endotoxin induced lung injury,[100;101] Therefore patients who need to be mechanically ventilated may benefit from the anti-inflammatory effects of isoflurane on cytokine release. Surprisingly, the effects of isoflurane on mechanical ventilation-induced pulmonary and systemic inflammation in healthy lungs has not been studied.

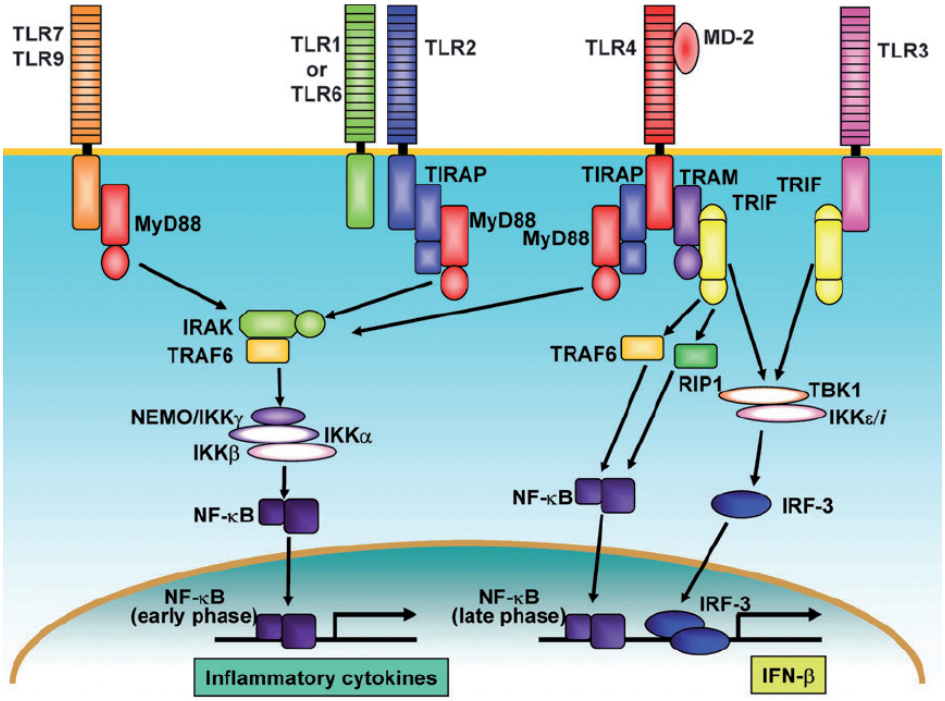

Figure 3: Dowstream signaling of Toll like receptors
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AIM OF THIS THESIS

Mechanical ventilation is associated with serious adverse effects. The injury caused by mechanical ventilation extends beyond the lungs and thus may be a major factor contributing to patient morbidity and mortality. Unravelling the inflammatory pathways activated by mechanical ventilation and investigating strategies to modulate this inflammatory response are the main aims of this thesis.

In **chapter 2** the effect of mechanical ventilation on inflammatory parameters in healthy animals was studied. Evidence is accumulating that mechanical ventilation can be the sole trigger for lung injury. An animal model was therefore developed using ventilatory protocols analogous to those currently used in clinical practice. To investigate whether mechanical ventilation alone can induce an inflammatory response without evoking histopathological damage, healthy C57BL6 mice were mechanically ventilated for up to four hours.

In **chapter 3** the mechanism underlying the inflammatory response following mechanical ventilation in the healthy lung was analyzed. TLR4 and TLR2 have been shown to mediate non-infectious lung injury, and might also be important in the development of VILI. We therefore investigated the role of TLR4 and TLR2 receptor signaling in the mechanical ventilation-induced inflammatory response in wild type, TLR4 knock out and TLR2 knock out mice. In addition, we searched for the presence of endogenous ligands for TLR4 in the lungs of these animals.

In **chapter 4,** the activation of the pathway downstream of TLRs following mechanical ventilation in the healthy lung was studied. TRIF acts downstream of TLR3 and 4 (figure 3) and has been identified as the key signaling pathway in acid induced lung injury. We mechanically ventilated wild type- and TRIF mutant mice to investigate the role of the TRIF pathway in the inflammatory response following mechanical ventilation.

Predominantly protective but also harmful effects of hypercapnia have been reported in experimental studies. In **chapter 5** the effect of hypercapnic acidosis on mechanical ventilation-induced inflammation in healthy lungs was investigated. Mice were mechanically ventilated using different inspiratory fractions of carbon dioxide.

Isoflurane is widely used in general anesthesia and has profound protective immunological effects on different organs, including the lung. In **chapter 6** the effect of isoflurane on the inflammatory response induced by mechanical ventilation in the healthy mouse lung was studied. Mechanically ventilated mice received different inspiratory concentrations of isoflurane during ventilation.

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Chapter 2

Mechanical ventilation in healthy mice induces reversible pulmonary and systemic cytokine elevation with preserved alveolar integrity:

An in-vivo model using clinical relevant ventilation settings

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ABSTRACT

Background:

Mechanical ventilation (MV) may activate the innate immune system, causing the release of cytokines. The resulting pro-inflammatory state is a risk factor for ventilator-induced lung injury. Cytokine increase results from direct cellular injury, but may also result from cyclic stretch alone as demonstrated *in-vitro*: mechanotransduction. To study mechanotransduction *in-vivo*, the authors used an animal MV model with clinically relevant ventilator settings, avoiding alveolar damage.

Methods:

Healthy C57BL6 mice (n=82) were ventilated (tidal volume, 8 ml/kg; positive end-expiratory pressure, 4 cm H_2O; fraction of inspired oxygen, 0.4) for 30, 60, 120 and 240 min. Assigned animals were allowed to recover 2 days after MV. Both pulmonary tissue and plasma interleukin (IL)-1α, IL-1β, tumor necrosis factor α, IL-6, IL-10, and keratinocyte-derived chemokine levels were measured. Histopathologic appearance of lung tissue was analyzed by light microscopy and electron microscopy.

Results:

In lung tissue, all measured cytokines and keratinocyte-derived chemokine levels increased progressively with MV duration. Light microscopy showed increased leukocyte influx but no signs of alveolar leakage or albumin deposition. Electron microscopy revealed intact epithelial cell and basement membranes with sporadically minimal signs of partial endothelial detachment. In plasma increased levels of IL-1α, tumor necrosis factor α, IL-6, and keratinocyte-derived chemokine were measured after MV. In the recovery animals, cytokine levels had normalized and no histologic alterations could be found.

Conclusions:

Mechanical ventilation induces reversible cytokine increase and leukocyte influx with preserved tissue integrity. This model offers opportunities to study the pathophysiological mechanisms behind ventilator-induced lung injury and the contribution of MV to the "multiple hit" concept.

INTRODUCTION

Mechanical ventilation (MV) is widely used in general anesthesia and is a lifesaving intervention in critically ill patients. It can, however, induce lung injury in the healthy lung or exacerbate damage in the already injured lung. This has been termed v*entilator-induced lung injury* (VILI).[1;2] Clinical studies show that the use of large tidal volumes ($V_T \geq 12$ -15 ml/kg) is associated with a poor prognosis; however, a "lung-protective ventilation strategy" (low tidal volumes [$V_T <$ 10 – 12 ml/kg], optimizing positive end-expiratory pressure [PEEP]) reduces but cannot prevent VILI.[1;3-8] VILI is characterized by the release of inflammatory mediators (especially cytokines), infiltration of leukocytes, alveolar and interstitial edema, alveolar protein deposition, cellular necrosis, and tissue disruption.[9;10] It is now commonly accepted that increased production of cytokines, especially interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor (TNF)-α, plays a key role in initiating or perpetuating lung injury.[11-17]
The clinical relevance of cytokine up-regulation by MV is the resulting proinflammatory state, because this makes the host more vulnerable to a "second hit" (*e.g.*, major surgery).[18] To note, MV itself can be the "second hit" where an already comprised host exists (*e.g.*, MV in the critically ill patient).[3;4;19]

Two mechanisms are believed to be responsible for MV-induced cytokine release. The first is direct trauma to the cell with disruption of the membranes, resulting in translocation of cytokines into both the alveolar space and the systemic circulation. This "decompartmentalization" has been demonstrated *in-vivo*.[13;20;21] The second has been termed *mechanotransduction*. *In vitro* studies show that most pulmonary cells, such as alveolar macrophages, epithelial cells, and endothelial cells, can produce cytokines in response to cyclic stretch.[22-24] However, the sensing mechanism of these physical forces and the translation into intracellular signals is largely unknown.[25]

In many of the currently available experimental VILI models, injurious MV settings (*e.g.*, $V_T >$ 25 ml/kg or peak pressures > 20-40 cm H_2O) have been used in healthy animals,[11;12;14;26;27] or the "multiple-hit" model was used by applying MV in already injured animals.[13;15;16;28;29] From these study designs, it is not possible to differentiate whether the observed increase in cytokine levels is the result of decompartmentalization, mechanotransduction, or both.

For a better understanding of the relevant pathophysiological mechanisms leading to VILI, it is important to study *in vivo* the effects of ventilation in the healthy lung, using ventilatory protocols analogous to those currently used during general anesthesia and in the intensive care unit patient. We studied the effects of MV in healthy mice, carefully searched for pulmonary cell or tissue disruption, counted leukocyte numbers and measured cytokine production in lung tissue and plasma.

MATERIAL AND METHODS

All experiments were approved by the Regional Animal Ethics Committee in Nijmegen, The Netherlands, and performed under the guidelines of the Dutch Council for Animal Care and the National Institutes of Health.

Animals:

Experiments were performed in male C57BL6 mice (n = 82; Charles River, Sulzfeld, Germany) aged 10 - 12 weeks, with weights ranging from 23 to 28 g.

Mechanical Ventilation in mice:

Mice were anesthetized with an intraperitoneal injection of a combination of ketamine, medetomidine and atropine (KMA): 7.5 µl per gram of body weight of induction KMA mix (consisting of 1.26 ml ketamine, 100 mg/ml; 0.2 ml medetomidine, 1 mg/ml; 1 ml atropine, 0.5 mg/ml; and 5 ml NaCl 0.9%) was given just before intubation. Animals were orally intubated under direct vision with an endotracheal tube (0.82 mm ID, 1.1 mm OD, length 25 mm). Endotracheal tube position was confirmed by end-tidal carbon dioxide analysis, using mass-spectrometry. Subsequently animals were connected to the ventilator (*MiniVent*®, Hugo Sachs Elektronik-Harvard apparatus, March-Hugstetten, Germany). V_T was set at 8 ml/kg and frequency at 150/min, which is well within the range of measured V_T and respiratory rate during spontaneous ventilation in C57BL6 mice.[30] All animals received 4 cm H_2O PEEP. To avoid direct oxygen toxicity, as reported by several authors,[31;32] the fraction of inspired oxygen (FiO_2) was set at 0.4.

To maintain anesthesia, 5.0 µl per gram of body weight boluses of maintenance KMA mix (consisting of 0.72 ml ketamine, 100 mg/ml; 0.08 ml medetomidine, 1 mg/ml; 0.3 ml atropine, 0.5 mg/ml; and 18.9 ml NaCl 0.9%) were given, *via* an intraperitoneally placed catheter, every 30 min. Throughout the experiment rectal temperature was monitored and maintained between 36° and 37.5°C using a heating pad.

Study groups:

Animals were divided into seven groups. Group C (n = 9) served as control group: after induction of anesthesia, these mice were killed immediately, without being ventilated. Animals in groups 30 (n = 6), 60 (n = 6), 120 (n = 9), 240 (n = 9), were ventilated for 30, 60, 120 and 240 min, respectively, and were killed immediately thereafter. In group R (recovery group), animals (n = 6) were extubated after being ventilated for 240 min and sacrificed after two days recovery. Anesthesia was discontinued in the group R animals 1 h before extubation. Group D (depleted group) animals (n = 6) were first leukocyte depleted by administering cyclophosphamide as described earlier.[33;34] These animals were then ventilated for 240 min and killed immediately thereafter.

A separate set of experiments (IABP group, n = 15) was conducted to assess if the chosen anesthetic and ventilation regime resulted in a stable and reproducible cardiorespiratory

condition. In these animals continuous intraarterial carotid blood pressure was measured. Arterial blood gas analysis was performed after 120 min (n=6) and 240 min (n=9). The same ventilator settings were used as for the mice in the aforementioned groups. We decided not to include the animals from the IABP group for the cytokine or histopathologic analysis to avoid possible interference with cytokine response resulting from instrumentation induced tissue damage.

In addition two control experiments were performed. In the first control experiment, animals (n=12) received the standard ventilation strategy (V_T, 8 ml/kg; PEEP, 4 cm H_2O; FiO_2 0.4). The lungs were removed after 0 min (n = 4, control), 120 min (n = 4) and 240 min (n = 4) of MV to measure wet/dry ratios. In the second control experiment animals (n = 4) were ventilated with a V_T of 16 ml/kg, PEEP of 4 cm H_2O and FiO_2 of 0.4 for 240 min. The lungs of these mice were used to histopathologically asses the effects of high-V_T ventilation in our model.

Material harvesting:

After the animals were killed, blood was collected by exsanguination, centrifuged at 14,000 rpm (13,000 g) (Eppendorf 5415 C, Nethler-Hinz GmbH, Hamburg, Germany) for 2 min and plasma was stored at - 80°C. Immediately after exsanguination, the heart and lungs were carefully removed *en block via* midline sternotomy. The right middle lobe was fixed for light microscopy (LM) and electron microscopy (EM), except in animals analyzed for wet/dry ratio. The remaining lung tissue was homogenized for the determination of cytokine concentrations.

Preparation and analysis of lung tissue:

For LM the material was fixed in 4% buffered formalin solution overnight at room temperature, dehydrated, and embedded in paraplast (Amstelstad, Amsterdam, The Netherlands). Sections of 4 μm-thickness were used for further analysis. The enzyme activity of leukocytes was visualized by enzyme histochemistry using chloracetatesterase staining (Leder). Periodic acid-Schiff staining was performed to analyze for alveolar albumin presence. Leukocytes were counted manually (20 fields per mouse), and after automated correction for air/tissue ratio, leukocytes per μm^2 were calculated.

For EM the material was fixed in 2.5% glutaraldehyde dissolved in 0.1 M sodium cacodylate buffer, pH 7.4, overnight at 4°C and washed in the same buffer. The tissue fragments were postfixed in cacodylate-buffered 1% OsO_4 for 120 min, dehydrated, and embedded in Epon 812 (Merck, Darmstadt, Germany). Ultrathin sections were cut on an Ultratome (Leica, Reichert Ultracuts, Vienna, Austria), and contrasted with 4% uranyl acetate for 45 min and subsequently with lead citrate for 4 min at room temperature. Sections were examined in a Jeol 1200 EX2 electron microscope (JEOL, Tokyo, Japan). The evaluating pathologist was blinded for the group and ventilation protocol to which the animal had been assigned.

For wet/dry ratios, both lungs were used; ratios were calculated by measuring lung weight before and after heating for 24 hours in a stove at 50°C.

Laboratory tests:

IL-1α and IL-1β were assessed using specific radioimmunoassays, as described previously.[35] Levels of TNF-α, IL-6, IL-10, and keratinocyte-derived chemokine (KC) in lung homogenate and plasma were measured using enzyme-linked immunosorbent assay (for TNF-α, IL-6 and IL-10: CytoSet, BioSource, Camarillo, CA; for KC: ELISA-Kit, R&D Systems, Minneapolis, MN). Lower detection limits were as follows: IL-1α and IL-1β: 40 pg/ml; TNF-α: 32 pg/ml; IL-6: 160 pg/ml; IL-10: 16 pg/ml; and KC: 160 pg/ml. For the assessment of KC in plasma in group 60 insufficient plasma was available; the plasma had to be diluted for analysis which increased the detection limit to 1,600 pg/ml. To investigate whether lipopolysaccharide contamination was present in our experimental setting we measured lipopolysaccharide in air, tubing, and the ventilator by Limulus Amebocyte Lysate testing (Cambrex Bio Science, Walkersville, MD; detection limit: 0.06 IU/ml).

Statistical Analysis:

Data are expressed as means (SD) when distributed normally (leukocyte counts and wet/dry ratios) and expressed as median (range) otherwise (cytokine concentrations). Statistical analysis was performed with SAS (SAS Institute Inc. Cary NC) statistical procedures. Because cytokine concentrations are not normally distributed, Kruskal-Wallis procedures were used, with *post hoc* comparisons of subgroups (Duncan). Data of a particular cytokine concentration variable were ranked, followed by analysis of variance in the General Linear Models procedure using the MEANS procedure with the Duncan option and Bonferroni correction for multiple comparisons. For the analysis of leukocyte counts and wet/dry ratios analysis of variance was used on non-ranked data with *post hoc* comparison of group means (Duncan). The level of significance was set at $P < 0.05$.

RESULTS

Cardiorespiratory Parameters:

The animals with an intraarterial canula (IABP-group) exhibited stable hemodynamic parameters throughout the experiments. Mean arterial pressure was within normal limits and remained above 65 mmHg in all animals. Blood gas analysis showed normal pH, arterial carbon dioxide tension ($PaCO_2$) and arterial oxygen tension (PaO_2) levels with a small decrease in base excess (table 1). Two of six animals in group R (recovery group) died directly after extubation. Before extubation these animals did not differ from surviving subjects in cardiorespiratory parameters. The remaining four animals were stable during the ventilation free interval, with normal activity and behavior and no respiratory distress or weight loss.

Histological Examination:

Electron microscopy examination of the lung tissue from animals in groups 30, 60, 120, and 240 revealed intact basement membranes and no signs of alveolar flooding. Type I pneumocytes

Table 1. Intraarterial Blood Pressure and Arterial Blood Gas Analysis during Mechanical Ventilation

		Blood Gas Values				
Duration of MV, min	MAP, mmHg	pH	PaO_2 (mmHg)	$PaCO_2$ (mmHg)	HCO_3 (mmol/l)	BE
0	102 (10)					
60	89 (12)					
120	83 (14)	7.36 (0.06)	229 (50)	38 (7)	20.4 (1.2)	-4.4 (1.5)
180	78 (6)					
240	79 (8)	7.35 (0.07)	194 (50)	36 (8)	19.6 (3.1)	-6.3 (2.0)

Values are mean (SD).
BE = base excess; MAP = mean arterial pressure; MV = mechanical ventilation; $PaCO_2$ = arterial carbon dioxide tension; PaO_2 = arterial oxygen tension.

sporadically showed signs of minimal membrane disruption and small partial detachment of endothelium (figs. 1A and B). Animals that were allowed to recover (group R) and unventilated animals (group C) showed no signs of membrane disruption or detachment of endothelium (fig. 1C). The four animals in the control experiment that were ventilated with a V_T of 16 ml/kg

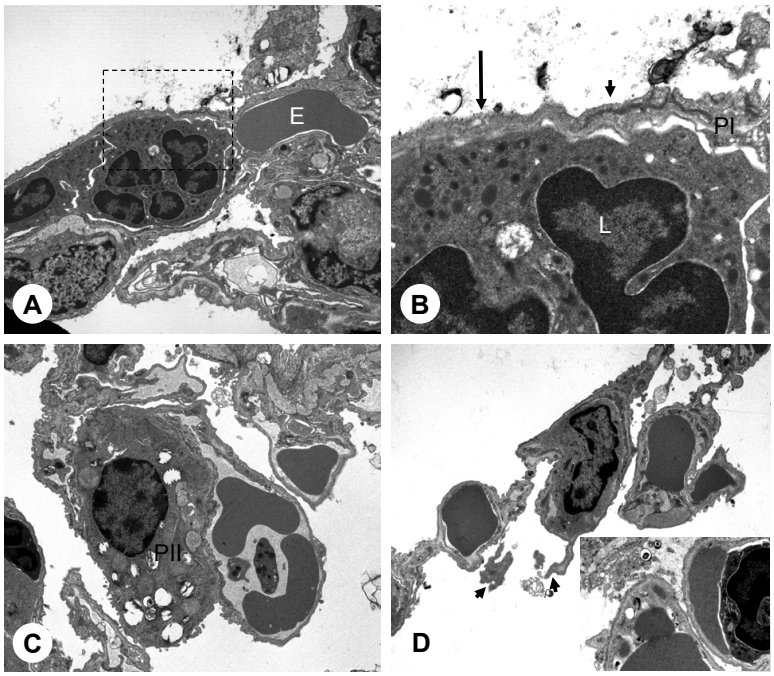

Fig 1. Electron microscopy.
(*A* and *B*) Lung tissue of healthy animals after 240 min of mechanical ventilation. *B* is a magnification of a section of *A* (see *dotted rectangle*). Mostly the membrane of type I pneumocyte stayed intact (*black arrowhead*, *B*); sporadically, signs of membrane disruption and partial detachment of endothelium occurred (*black arrow*, *B*). Animals that were allowed to recover for two days (group R) and unventilated healthy control animals (group C) showed no signs of membrane disruption and no detachment of endothelium (*C*). Animals ventilated with tidal volume of 16 ml/kg showed extensive damage with lungs appearing overinflated with loss of septal walls (*black arrowheads*, *D*) and injury of type I pneumocyte and endothelium (*inset*, *D*). Magnification: *A* and *C*: 5,000x; *B*: 15,000x; *D*: 3,000x (*inset* 7,000x). E = erythrocyte; L = leukocyte; PI = type I pneumocyte; PII = type II pneumocyte.

showed significant injury; lungs appeared overinflated (airtrapping) with loss of septal walls and injury of type I pneumocyte (fig. 1D).

Light microscopy examination using periodic acid-Schiff staining showed no intraalveolar albumin. Leder staining revealed a substantially higher number of pulmonary leukocytes after 120 and 240 min of MV (fig. 2). No differences in leukocyte counts were found in the animals that were allowed to recover (group R) compared with the unventilated animals (group C).

Wet/dry ratios showed increased ratios only after 240 min of MV. Data are presented in table 2.

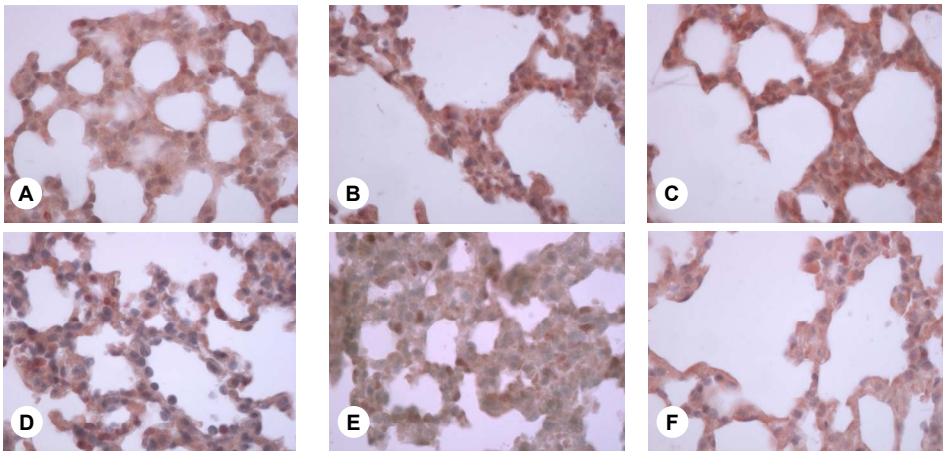

Fig. 2: Light microscopy.
Light microscopy examination of lung tissue after Leder staining revealed a significantly higher number of pulmonary leukocytes in healthy animals after 120 (group 120, *D*) and 240 min (group 240, *E*) of mechanical ventilation compared with the unventilated control animals (group C, *A*). Significantly lower numbers of pulmonary leukocytes were found in the animals that were allowed to recover (group R, *F*) compared with animals ventilated for 240 min (group 240, *E*). No differences were found between unventilated controls (group C, *A*) and the animals that were allowed to recover (group R, *F*). For the results of leukocyte counts, see table 2. (*A*) Unventilated control animals. (*B-E*) Healthy animals receiving mechanical ventilation for 30, 60, 120 and 240 min. (*F*) Animals that were allowed to recover for 2 days after being ventilated for 240 min (group R). Magnification: 750x.

Table 2. Leukocyte Counts and Wet/Dry Ratios

Groups	Leukocytes x 10-4 / μm^2, Mean (SD)	P value	Wet/Dry Ratio, Mean (SD)	P value
Control	2.1 (1.3)		4.68 (0.014)	
"30"	2.4 (1.6)	NS		
"60"	2.2 (2.2)	NS		
"120"	9.7 (5.0)	<0.05	4.81 (0.13)	NS
"240"	5.7 (3.1)	<0.05	5.01 (0.06)	<0.05
Recovery	1.2 (0.9)	<0.05*		

Values are mean (SD).
P values compared with Control group (unventilated animals). * *P* value compared to group 240 (240 min of ventilation).
NS = not significant.

Cytokine Concentration Induced by Mechanical Ventilation:

Mechanical ventilation with a V_T of 8 ml/kg, PEEP of 4 cm H_2O and FiO_2 of 0.4 resulted in a significant increase in IL-1α, IL-1β, TNF-α, IL-6, IL-10, and KC in lung tissue homogenate when compared with unventilated animals (group C). These cytokine concentrations increased with the duration of MV, with KC being the first to increase from 30 min of MV onwards. IL-1β, TNF-α, IL-6 and IL-10 levels increased after 60 min, while IL-1α increased after 240 min compared with group C animals (fig. 3).

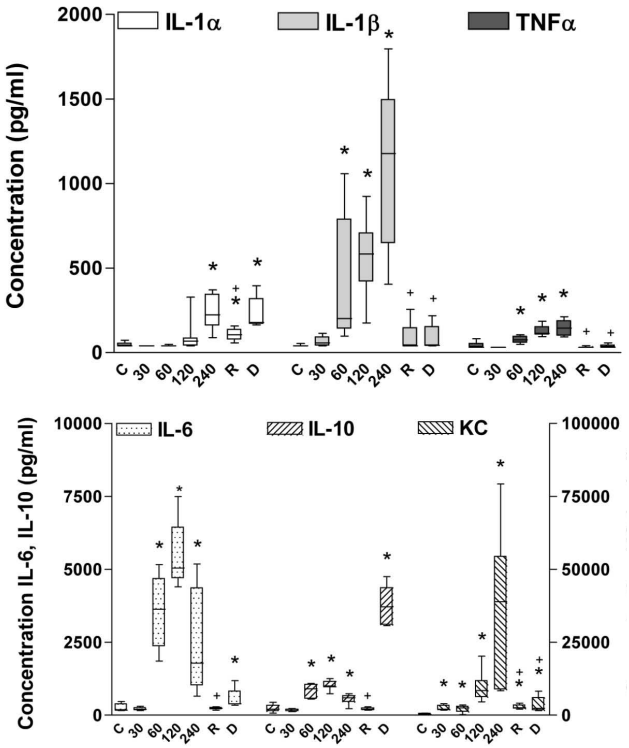

Fig. 3: Cytokine levels in lung tissue homogenate.
Mechanical ventilation significantly increased levels of interleukin (IL)-1α, IL-1β, tumor necrosis factor (TNF)-α, IL-6, IL-10 and keratinocyte-derived chemokine (KC) in lung tissue homogenate compared with unventilated animals (group C). When animals were allowed to recover for 2 days (group R), all lung tissue cytokine levels were found to be lower when compared with levels found in animals killed immediately after 240 min of ventilation (group 240). IL-1α and KC levels in group R animals were higher compared with group C animals. In leukocyte-depleted animals (group D), mechanical ventilation resulted in significantly higher levels of lung tissue IL-1α, IL-6, IL-10 and KC compared with group C animals. IL-1β, TNF-α and KC levels were lower compared with the levels found after 240 minutes of MV in healthy animals (group 240). Data are expressed as *box* (median, 25th and 75th percentiles) and *whiskers* (range). * $P < 0.05$ compared with control; + $P < 0.05$ compared with group 240 (= 240 min of ventilation), – = Lower detection limit; 30, 60, 120, 240 = minutes of ventilation in healthy mice; C = control group (unventilated, healthy mice); D = leukocyte-depleted group (these animals were leukocyte depleted before 240 min of mechanical ventilation and were killed immediately thereafter); R = recovery group (these animals were ventilated for 240 min and analyzed after 2 days recovery).

In plasma, TNF-α, IL-6 and KC levels were elevated from 120 min onward compared with group C animals, and IL-1α after 240 min of MV. IL-1β and IL-10 levels were not different from those of the group C animals (fig. 4).

When animals were allowed to recover for 2 days (group R), after being ventilated for 240 min, all lung tissue cytokine levels were lower compared with levels found in animals killed

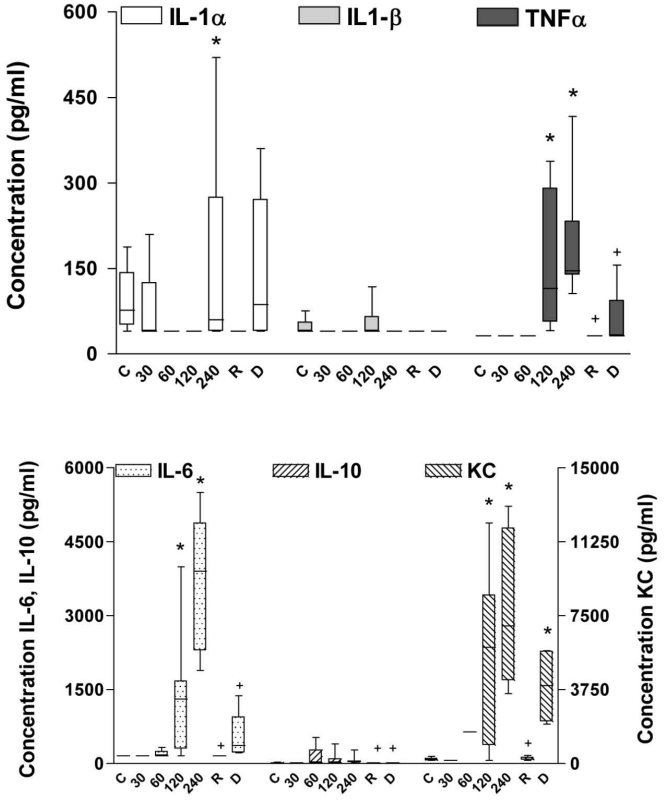

Figure 4: Cytokine levels in plasma.
Mechanical ventilation significantly increases tumor necrosis factor (TNF)-α, interleukin (IL)-6 and keratinocyte-derived chemokine (KC) plasma levels from 120 min onward compared with unventilated animals (group C); IL-1α level was increased only after 240 min. When animals were allowed to recover for 2 days (group R) TNF-α, IL-6, IL-10, and KC were found to be lower when compared with levels found in animals killed immediately after 240 min of ventilation (group 240). In leukocyte-depleted animals (group D), mechanical ventilation resulted in significantly higher levels of plasma levels for KC compared with group C animals. TNF-α, IL-6, and IL-10 plasma levels were lower compared with the levels found after 240 min of mechanical ventilation in healthy animals (group 240). Data are expressed as *box* (median, 25th and 75th percentiles) and *whiskers* (range). * $P < 0.05$ compared with control. + $P < 0.05$ compared with group 240 (= 240 min of ventilation). – = Lower detection limit; 30, 60, 120, 240 = minutes of ventilation in healthy mice; C = control group (unventilated, healthy mice); D = leukocyte-depleted group (these animals were leukocyte depleted before 240 min of mechanical ventilation and killed immediately thereafter). R = recovery group (these animals were ventilated for 240 min and analyzed after 2 days recovery).

immediately after 240 min of ventilation (group 240). IL-1α and KC levels in group R animals were higher compared with group C animals. Plasma levels of TNF-α, IL-6, IL-10 and KC were found to be lower when compared with group 240.

The Effect of Leukocyte Depletion on the Release of Cytokines:

In leukocyte-depleted animals (group D), MV resulted in significantly higher levels of lung tissue IL-1α, IL-6, IL-10, and KC compared with group C animals. IL-1β, TNF-α, and KC levels were lower compared with the levels found after 240 min of MV in healthy (non-leukocyte depleted) animals (group 240). MV resulted in higher plasma levels of KC compared with group C animals. TNF-α, IL-6, and IL-10 levels in plasma were lower compared with the levels found in group 240.

No lipopolysaccharide could be detected in our experimental setting.

DISCUSSION

The current study demonstrates that MV in healthy mice using clinically relevant ventilator settings with low V_T preserves alveolar integrity but induces reversible cytokine increase and leukocyte influx. This rapid increase in cytokine levels and leukocyte influx, however, does not result in persistent inflammation and VILI.

Our findings suggest that "noninjurious" or "lung-protective" ventilation does not exist and that even this careful mode of ventilation strategy leads to a reversible inflammatory response. Fortunately, MV in elective, healthy patients rarely leads to clinical significant injury. Apparently, in most circumstances the lung is able to cope with the MV-induced inflammatory reaction. This is demonstrated in a clinical study by Plotz *et al*.[36] which showed that 2 h of MV (V_T of 10 ml/kg) in healthy children, anesthetized for cardiac catheterization, resulted in elevated alveolar IL-6 and TNF-α concentrations without clinical signs of pulmonary dysfunction. In contrast with this are the findings in the clinical study of Wrigge *et al*.[37] who found no ventilation-induced increase in cytokines. However, in this study the ventilation duration was limited to one hour and cytokines were only measured in plasma and not in the lung.

The current study is essentially different from previous experimental studies. In those studies either injurious MV settings with large tidal volumes and high peak inspiratory pressures were used,[11;12;14;26;27;38] or lungs were preinjured using lipopolysaccharide, hydrochloric acid or surfactant depletion.[13;15;16;28;29] Another major difference is that cytokine levels were measured in lung lavage fluid, whereas we used lung tissue homogenate.[11;12;14;38] Lung lavage - by itself potentially injurious as it is often performed using large volumes[11;12;38] requires cellular and alveolar leakage for the detection of cytokines. Analysis of lung tissue homogenate allows detection of cytokines before appearance in the alveolar space. This method of material harvesting does not allow the identification of the specific cells responsible for the measured

increases in cytokine levels. However, the observed increase in KC after 30 min of MV, before the leukocyte influx, suggests a primary pulmonary origin of this cytokine. This is further supported by the finding of KC elevation in the leukocyte-depleted animals (group D). However, we did not study the possible effects of cyclophosphamide on cytokine synthesis of pulmonary cells.

In this study, EM analysis revealed that our MV mode almost completely retained histologic integrity, with only sporadically minimal changes in a few samples. Most importantly, basement membranes were not disrupted, signifying alveolar integrity. Although LM has been used for quantitative analysis of leukocytes and can provide some qualitative evidence of lung injury (*i.e.*, alveolar flooding, thickening of alveolar septa),[39;40] more detailed evaluation of structural changes in the lung requires EM.[41] The effects of large V_T in the animals ventilated with V_T of 16 ml/kg was clearly visible with EM as damage with loss of compartmentalization, consistent with findings in a previous publication.[9] We observed increased wet/dry ratios in animals after 240 min of MV. However, we conclude that this increased wet/dry ratio (without any other signs of possible lung damage as shown by EM and LM examination) is not of clinical significance. This is supported by the finding of the complete recovery of the animals who were allowed to recover after 240 min of ventilation (group R).

Factors affecting cytokine response other than MV were carefully avoided. The possibility of triggering an inflammatory response by invasive procedures (*i.e.*, insertion of an intraarterial line),[18] was eliminated by performing our experiments in non-invasively monitored animals, after having documented cardiorespiratory stability in invasively monitored animals (IABP-group). A limulus amebocyte lysate test excluded possible aerogenic lipopolysaccharide contamination during our experiments. Cardiorespiratory parameters and the choice of the anesthetics are known to influence the cytokine profile. In the current study mean arterial pressure was maintained above 65 mmHg and blood gas analysis showed normal pH, $PaCO_2$ and PaO_2 levels. Only a small decrease in base excess after 120 and 240 min of MV was observed, comparable with other studies.[11;12] The slight decrease in base excess in the presence of a normal mean arterial pressure unlikely interferes with our observations. The effect of anesthetics on hemodynamic stability in mice has been studied extensively by Zuurbier *et al*.[42] who found KMA mix superior compared with other regimes (*e.g.*, fentanyl – fluanisone – midazolam mix or isoflurane). Some anesthetics, *e.g.*, propofol,[43] volatile anesthetics,[44;45] and ketamine,[46;47] are known to influence cytokine profiles. Ketamine is known to have an inhibitory effect on lipopolysaccharide-induced cytokine production.[46;48-50] In the current study all animals received the KMA mix. Ideally, an additional control group of spontaneously breathing animals under KMA-anesthesia is needed. However, this will result in hypoventilation with severe respiratory acidosis and hemodynamic instability. Two mice in group R (recovery group) died immediately following extubation; the cause of death was due to airway problems related to residual effects of the anesthesia. The other mice in group R made uneventful recoveries. By excluding these confounding factors, we attribute the increase in cytokine levels to MV.

Therefore, even low-V_T MV induces an inflammatory response and, in a "multiple-hit" situation, might be the additional "proinflammatory" hit resulting in lung injury. Modulation of the inflammatory response may offer strategies to reduce VILI. In this respect anesthetics may play a role because volatile anesthetics have been shown to exhibit antiinflammatory effects in different organ systems and might be able to modulate the release of cytokines.[51] The influence of different anesthetics on the inflammatory response in our model needs further investigation. Recently Jiang *et al*.[52] discovered that Toll-like receptor-4 mediated inflammation by endogenous compounds might also be important in the development of VILI. Further study of the Toll-like receptors and the molecules with which they interact may reveal more insight into the molecular mechanisms of VILI.

CONCLUSION

The current study shows that in healthy male mice, a short period of "noninjurious" ventilation induces a reversible inflammatory reaction, while preserving tissue integrity. This model offers opportunities to study the pathophysiological mechanisms of VILI and the contribution of MV to the "multiple-hit" concept.

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Chapter 3

Low tidal volume mechanical ventilation induces a Toll-like receptor 4 dependent inflammatory response in healthy mice.

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ABSTRACT

Background:

Mechanical ventilation (MV) can induce ventilator-induced lung injury. A role for pro-inflammatory pathways has been proposed. The present studies analyzed the roles of Toll-like receptor (TLR)-4 and TLR2 involvement in the inflammatory response following MV in the healthy lung.

Methods:

Wild type (WT) C57BL6, TLR4 knock out (KO) and TLR2 KO mice were mechanically ventilated for 4 hours. Broncho-alveolar lavage fluid was analyzed for presence of endogenous ligands. Lung homogenates were used to investigate changes in TLR4 and TLR2 expression. Cytokines were measured in lung homogenate and plasma and leucocytes were counted in lung tissue.

Results:

MV significantly increased endogenous ligands for TLR4 in broncho-alveolar lavage fluid and relative mRNA expression of TLR4 and TLR2 in lung tissue. In lung homogenates, MV in WT mice increased levels of keratinocyte-derived chemokine (KC), interleukin (IL)-1α and IL-1β. In TLR4 KO mice MV increased IL-1α, but not IL-1β and the increase in KC was less pronounced. In plasma, MV in WT mice increased levels of IL-6, KC and tumor necrosis factor-α. In TLR4 KO mice MV did not increase levels of IL-6 and tumor necrosis factor-α and the response of KC was less pronounced. MV in TLR2 KO mice did not result in different cytokine levels compared with WT mice. In WT and TLR2 KO, but not in TLR4 KO mice, MV increased the number of pulmonary leukocytes.

Conclusions:

The current study supports a role for TLR4 in the inflammatory reaction following short-term MV in healthy lungs. Increasing the understanding of the innate immune response to MV may lead to future treatment advances in ventilator-induced lung injury, in which TLR4 may serve as a therapeutic target.

INTRODUCTION

Mechanical ventilation (MV) facilitates surgical interventions under general anesthesia and is a life saving intervention in acute respiratory failure. However, MV can induce lung injury; this has been termed ventilator-induced lung injury (VILI).[1] VILI can exacerbate damage in the already injured lung but can also occur in healthy lung.[2] The mechanisms of VILI are incompletely understood but a role for pro-inflammatory pathways has been proposed.[3]

Animal models have been used to study the pathophysiology underlying VILI. Traditionally, in these models lungs were preinjured, for instance by lipopolysaccharide.[4] Lipopolysaccharide is a component of the outer membrane of Gram-negative bacteria. Upon binding to Toll-like receptor (TLR)-4,[5] lipopolysaccharide induces an intense inflammatory response leading to lung injury.[6-8] TLRs are essential in host defense against pathogens by virtue of their capacity to recognize various microbes and initiate an immune response.[9]

To date, 12 TLRs have been identified in mammals.[5] Recent studies indicate that TLRs not only recognize microbial products, but also endogenous ligands released from damaged tissue, the so-called 'danger signals'.[10;11] Non-infectious lung injury induced by hemorrhage,[12-14] ischemia-reperfusion,[15] contusion,[16] hyperoxia or administration of bleomycin,[17] resulted in inflammatory reactions via TLR4 and/or TLR2 signaling supporting the existence of endogenous ligands. Recently, endogenous ligands for TLR4 and TLR2 were identified, including heat shock protein 60,[18] fibronectin,[19] heparan sulfate proteoglycan,[20] biglycan,[21] hyaluronan[22] and the myeloid-related proteins 8 and 14.[23]

The concept of TLR mediated inflammation by endogenous ligands is interesting and may be involved in the development of VILI in the normal lung. Recently, we have shown that low tidal volume MV in healthy mice induces a transient inflammatory response without altering the structural integrity of the lung.[24] The aim of the present study was to test the hypothesis that TLR4 and/or TLR2 receptor signaling is involved in the inflammatory response induced by MV in the healthy lung. To test this hypothesis wild type (WT), TLR4 knock out (KO) and TLR2 KO mice were mechanically ventilated.

MATERIAL AND METHODS

All experiments were approved by the Regional Animal Ethics Committee in Nijmegen and performed under the guidelines of the Dutch Council for Animal Care and the National Institutes of Health. The current study protocol was designed after a pilot study was performed.

Animals:

All experiments were carried out in adult male C57BL6 mice (n=64): TLR4 KO (C57BL6 background) mice (10-12 wk) (n=14; body weight 30 (±3) gram) and age matched wild type (WT)

mice (n=40; body weight 25 (±3) gram). In a separate set of experiment TLR2 KO (C57BL6 background) mice (22-24 wk) (n=5; body weight 30 (±2) gram) and age matched WT mice (n=5; body weight 33 (±2) gram) were used. All KO mice were extensively backcrossed (at least 10 times) and were a kind gift from professor Shizuo Akira M.D., Ph.D., Osaka University, Osaka, Japan. WT mice were purchased from Charles River, Sulzfeld, Germany.

Mechanical ventilation in mice:

Mice were anesthetized with an intraperitoneal injection of a combination of ketamine, medetomidine and atropine as described previously.[24] Mice in the unventilated groups were sacrificed immediately after induction of anesthesia. Mechanically ventilated (*MiniVent*®, Hugo Sachs Elektronik-Harvard apparatus, March-Hugstetten, Germany) animals were orally intubated, ventilated for 4 hours and sacrificed immediately thereafter. The following settings were used during controlled MV: tidal volume 8 ml/kg body weight, frequency 150/min, positive end-expiratory pressure 4 cm H_2O and fraction of inspired oxygen 0.4. Rectal temperature was monitored continuously and maintained between 36.0°C and 37.5°C using a heating pad.

Experimental design:

The first set of experiments was performed to investigate the presence of endogenous ligands for TLR4 in broncho-alveolar lavage (BAL) fluid. BAL was performed after 4 hours of MV (group V-WT, n=8) or directly after induction of anesthesia (group C-WT, n=8).

The second set of experiments was designed to investigate changes in pulmonary TLR4 and TLR2 expression in WT mice and to investigate changes in cytokine profile in wild type and TLR4 KO mice following MV. Blood and lungs were harvested after 4 hours of MV in WT (group V_{TLR4}WT, n = 8) and TLR4 KO mice (group V_{TLR4}KO, n = 8) or immediately after induction of anesthesia in WT (group C_{TLR4}WT, n = 8) and TLR4 KO mice (C_{TLR4}KO, n = 6).

In the third set of experiments the role of TLR2 in MV-induced inflammation was studied. Blood and lungs were harvested after 4 hours of MV in WT (group V_{TLR2}WT, n=5) and TLR2 KO mice (group V_{TLR2}KO, n=5).

Lipopolysaccharide was measured in air, tubing, ventilator and BAL fluid from unventilated animals (n=4) by Limulus Amebocyte Lysate testing (Cambrex Bio Science, Walkersville, USA; detection limit: 0.06 IU/ml) to rule out contamination with lipopolysaccharide in our experimental setting.

Tissue harvesting:

Blood was collected by exsanguination, centrifuged at 14,000 rpm / (13,000 g) (Eppendorf 5415 C, Nethler-Hinz GmbH, Hamburg) for 2 minutes and plasma was stored at -80°C. Immediately after exsanguination, heart and lungs were carefully removed *en block* via midline sternotomy. The right upper and lower lobe were snap frozen in liquid nitrogen and stored at -80°C. The

right middle lobe was fixed for light microscopy (LM). The left lung was homogenized for measurement of cytokine concentrations.

Broncho-alveolar lavage and analysis for the presence of endogenous ligands:

For the BAL procedure 250 μl of sterile saline (0.9%) of 37°C was injected and gently aspirated through the endotracheal tube. Lavage fluid was snap frozen in liquid nitrogen and stored at -80°C. Human embryonal kidney (HEK)-293-TLR4 cell lines were purchased from InvivoGen (San Diego, California, USA) and cultured according to the manufacturer's guidelines. For stimulation, 5.10^4 cells/well were used in flat-bottom 96-wells plates. HEK293-TLR4 cells were exposed to 50μL of BAL fluid and added to 200 μL culture medium for 24 hours. Subsequently, interleukin (IL)-8 was measured in culture supernatants using Luminex bead array technology. To verify the presence of endogenous TLR4 ligands, the BAL fluid was incubated with or without 10 μg/ml *B. quintana* lipopolysaccharide as a specific TLR4 antagonist.[25;26] To rule out lipopolysaccharide contamination, we performed the HEK-293-TLR4 assay in the presence and absence of polymyxin B (2 μg/mL) with BAL fluid from ventilated WT mice (n=4).

mRNA analysis of lung homogenates:

For Polymerase Chain Reaction (PCR) analysis of mRNA, the right upper and lower lobe were homogenized with a micro-dismembrator II (Braun, Melsungen, Germany). Total RNA was extracted in 1 ml TRIzol reagent. Subsequently 200 μL chloroform and 500 μl 2-propanol (Merck, Darmstadt, Germany) were used to separate the RNA from DNA and proteins. After a wash step with 75% ethanol (Merck, Darmstadt, Germany) the dry RNA was dissolved in 30 μL of water.

To obtain double strain cDNA, DNase treated total RNA 1 μg with oligo dT primers (0.01 μg/ml) was reverse transcribed in a RT-PCR with a total volume of 20 μl. Subsequently, quantitative PCR was performed using ABI/PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). PCR's of glyceraldehyde-3-phosphate dehydrogenase, TLR2 and TLR4 were performed with Sybr Green PCR Master Mix (Applied Biosystems, Foster City, CA), 5 μL 1/20 diluted cDNA and primers in a final concentration of 300 nmol/L in a total volume of 25 μL. The primers were developed using Primer Express® software (Applied Biosystems, Foster City, CA). Quantification of the PCR signals of each sample was performed by comparing the cycle threshold values (C_t), in duplicate, of the gene of interest with the C_t values of the glyceraldehyde-3-phosphate dehydrogenase housekeeping gene. TLR2 and TLR4 mRNA expression was expressed as relative expression to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase. All primers were validated according to the protocol and standard curves were all within the tolerable range.

Cytokine analysis in lung homogenates and plasma:

Tumor necrosis factor (TNF)-α, IL-6, IL-10, and Keratinocyte-derived chemokine (KC) in the homogenized left lung and in plasma were analyzed by enzyme-linked-immunosorbent assay (ELISA) (for TNF-α, IL-6 and IL10; CytoSet, BioSource, USA; for KC; ELISA-Kit, R&D, USA). IL-1α and

IL-1β were only assessed in lung homogenates, because of insufficient plasma, using specific radio-immuno-assays, as described previously.[27] Lower detection limits: IL-1α and IL-1β; 40 pg/ml, TNF-α; 32 pg/ml, IL-6; 160 pg/ml, IL-10; 16 pg/ml and KC; 160 pg/ml.

Histological examination:

The right middle lung lobe was fixed in 4% buffered formalin solution overnight at room temperature, dehydrated and embedded in paraplast (Amstelstad, Amsterdam, The Netherlands). Sections of 4 µm-thickness were used. The enzyme activity of leukocytes was visualized by enzyme histochemistry using chloracetatesterase staining (Leder staining). Leukocytes were counted manually (20 fields per mouse), and after automated correction for air/tissue ratio, the number of leukocytes/µm^2 was calculated.

Statistical Analysis:

Data are expressed as mean (SD) when distributed normally (body weight, endogenous ligands, relative mRNA expression, leukocyte counts) and expressed as median (range) otherwise (cytokine concentrations). Statistical analysis was performed with SAS (SAS Institute Inc. Cary NC, USA) statistical procedures. Since cytokine concentrations are not normally distributed, Kruskal Wallis procedures were used, with post hoc comparisons of subgroups (Duncan). Data of a particular cytokine concentration variable were ranked, followed by ANOVA in the General Linear Models procedure using the MEANS procedure with the Duncan option and Bonferroni correction for multiple comparisons. For analysis of endogenous ligands, relative mRNA expression and leukocyte counts ANOVA was used on non-ranked data with post hoc comparison of group means. The level of significance was set at $P < 0.05$.

RESULTS

Presence of endogenous ligands for TLR4 and TLR4/TLR2 mRNA expression after mechanical ventilation:

BAL fluid from unventilated WT mice resulted in increased production of IL-8 by HEK293-TLR4 cells compared with the medium. This could only be partly reduced by co-incubation with a highly specific TLR4 antagonist, indicating that these cells generate IL-8 in a TLR4 independent manner as well (see discussion, role for endogenous ligands). BAL fluid from ventilated WT mice induced a significantly higher IL-8 response compared with BAL from unventilated WT mice and co-incubation with a highly specific TLR4 antagonist did significantly reduce the IL-8 response (figure 1). From the experiments performed in the presence of TLR-4 antagonist, it can be derived that the TLR4 dependent response was approximately 5 fold higher in ventilated WT mice compared with unventilated WT mice.

MV increased the relative mRNA expression of TLR4 and TLR2 in lung tissue of ventilated WT mice as compared with unventilated WT mice (figure 2).

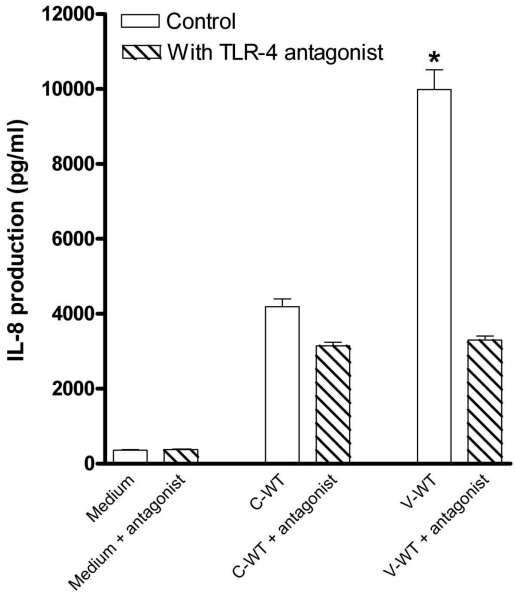

Figure 1: Presence of endogenous ligands for Toll-like receptor-4 (TLR4) in broncho-alveolar lavage fluid after 4 hours of mechanical ventilation.
Interleukin-8 production by human embryonal kidney-293-TLR4 reporter cells after exposure to broncho-alveolar lavage fluid. After 4 hours of mechanical ventilation endogenous ligands for TLR4 were enhanced in broncho-alveolar lavage fluid of the ventilated wild type mice (group V-WT) ($P < 0.0001$) compared with unventilated wild type mice (group C-WT). Addition of a highly specific TLR4 antagonist decreased the IL-8 production to the level found in unventilated wild type mice (group C-WT). Data expressed as mean (SD). * = $P < 0.05$ compared with group C-WT.

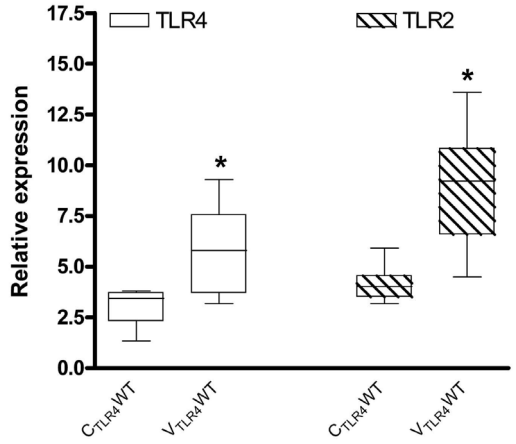

Figure 2: Pulmonary relative mRNA expression of Toll-like receptor (TLR) 4 and TLR2 after 4 hours of mechanical ventialtion.
Mechanical ventilation resulted in enhanced relative mRNA expression of TLR4 ($P = 0.008$) and TLR2 ($P = 0.0006$) in lung tissue of wild type mice (group V_{TLR4}WT) compared with unventilated wild type mice (group C_{TLR4}WT). Data are expressed as Box (median, 25th, 75 percentile) and Whiskers (range).
* = $P < 0.05$ compared with group C-WT.

Changes in cytokine concentration induced by MV:

LUNG HOMOGENATES: MV in WT mice increased levels of IL-1α, IL-1β and KC in lung homogenates (figure 3). However, in TLR4 KO mice MV did not increase IL-1β in lung homogenates and the MV-induced increase in KC was less pronounced. Interestingly, TLR4 KO did not reduce the response of IL-1α to MV. Neither MV, nor TLR4 KO did affect TNF-α, IL-6 and IL-10 concentrations in lung homogenates.

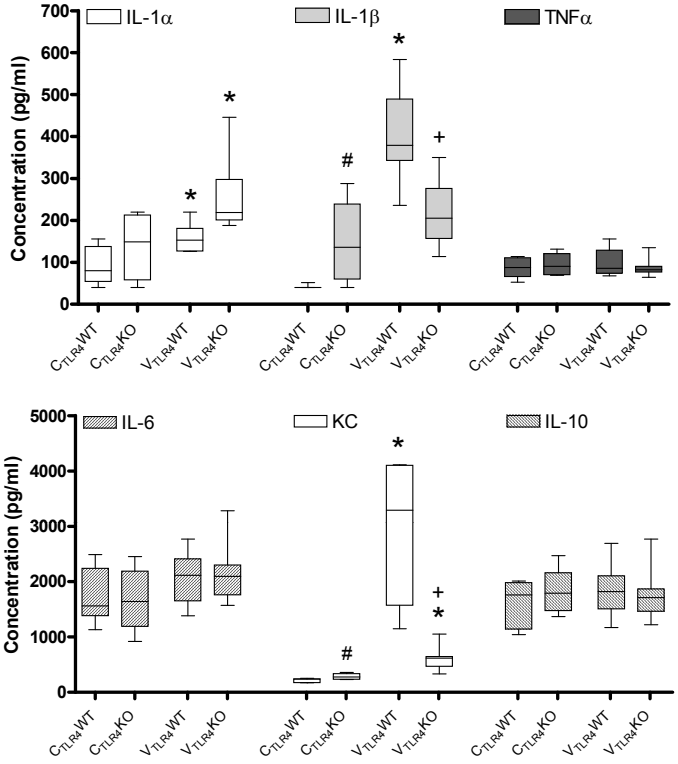

Figure 3: Cytokine levels in lung homogenates.
Levels of interleukin (IL)-6, keratinocyte-derived chemokine (KC), IL-10, IL-1α, IL-1β and tumor necrosis factor-α in unventilated (C) and ventilated (V) wild type (WT) and Toll-like receptor (TLR) 4 knock out (KO) mice. Mechanical Ventilation in WT mice (group V_{TLR4}WT) increased KC ($P < 0.0001$), IL-1α ($P = 0.003$) and IL-1β ($P < 0.0001$) in lung tissue homogenates when compared with unventilated WT mice (group C_{TLR4}WT). In TLR4 KO mice (V_{TLR4}KO) mechanical ventilation did not increase IL-1β in lung homogenates. Mechanical ventilation in TLR4 KO (V_{TLR4}KO) mice did increase levels of KC ($P = 0.0003$) and IL-1α ($P = 0.003$) when compared with unventilated TLR4 KO mice (group C_{TLR4}KO). Ventilated TLR4 KO mice (group V_{TLR4}KO) showed significantly lower levels of KC ($P < 0.0001$) and IL-1β ($P = 0.0005$) in lung homogenates compared with ventilated WT mice (group V_{TLR4}WT). Between unventilated animals the levels of KC ($P = 0.0069$) and IL-1β ($P= 0.048$) in lung homogenates where higher in the TLR4 KO mice (C_{TLR4}KO) compared with WT mice (C_{TLR4}WT). Data are expressed as Box (median, 25th, 75 percentile) and Whiskers (range). * = $P < 0.05$ compared with age matched unventilated mice. + = $P < 0.05$ compared with ventilated WT mice (V_{TLR4}WT). # = compared with unventilated WT mice (C_{TLR4}WT). - = lower detection limit.

PLASMA: In WT mice, MV was associated with significantly increased levels of IL-6, KC and TNF-α in plasma (figure 4). In TLR4 KO mice MV did not increase plasma levels of IL-6 and TNF-α. MV did result in increased levels of KC, although the response was less pronounced than in WT mice (figure 4).

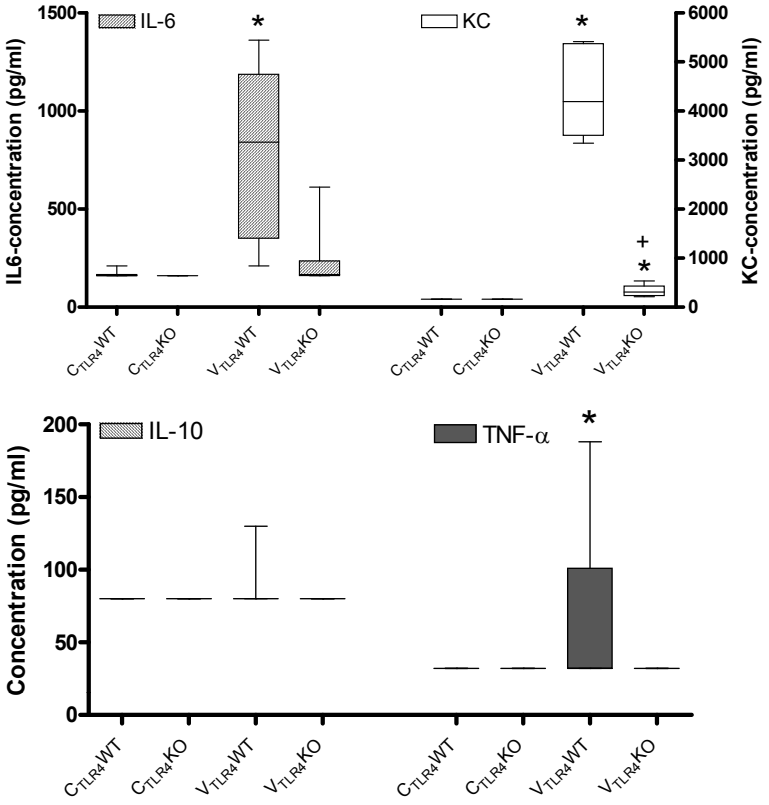

Figure 4: Cytokine levels in plasma.
Levels of interleukin (IL)-6, keratinocyte-derived chemokine (KC), IL-10 and tumor necrosis factor-α in unventilated (C) and ventilated (V) wild type (WT) and Toll-like receptor (TLR) 4 knock out (KO) mice. Mechanical ventilation in WT mice (group V_{TLR4}WT) increased IL-6 ($P < 0.0001$), KC ($p < 0.0001$) and tumor necrosis factor-α ($P = 0.04$) in plasma when compared with unventilated WT mice (group C_{TLR4}WT). In TLR4 KO mice (V_{TLR4}KO) MV did increase KC ($P < 0.0001$) in plasma when compared with unventilated TLR4 KO mice (group C_{TLR4}KO); however, this response was significantly lower ($P = 0.0002$) compared with the ventilated WT mice (group V_{TLR4}WT). No differences were found in the plasma levels of cytokines in the unventilated groups. The median of TNF alpha in the V-WT group is not visible because this coincided with the 25 percentile. Data are expressed as Box (median, 25th, 75 percentile) and Whiskers (range).
* = $P < 0.05$ compared with their own unventilated mice. + = $P < 0.05$ compared with ventilated WT mice (V_{TLR4}WT). - = lower detection limit.

MV in TLR2 KO mice did not result in statistically different cytokine levels in lung tissue homogenates and plasma compared with their age matched WT ventilated animals (figure 5 and 6).

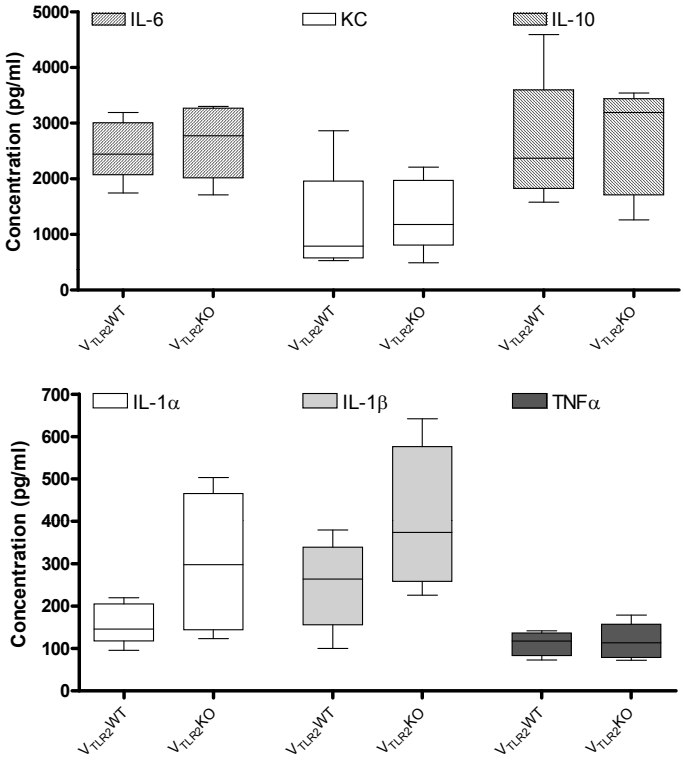

Figure 5: Cytokine levels in lung homogenates.
Levels of interleukin (IL)-6, keratinocyte-derived chemokine (KC), IL-10, IL-1α, IL-1β and tumor necrosis factor-α in ventilated (V) wild type (WT) and Toll-like receptor (TLR) 2 knock out (KO) mice. Mechanical ventilation in TLR2 KO mice did not result in statistically different cytokine levels in lung tissue homogenates compared with their age matched WT ventilated animals.

Histological examination:

In WT mice, MV increased the number of pulmonary leukocytes (table 1). In TLR4 KO mice MV did not affect the number of leukocytes in the lung. Unventilated TLR4 KO mice expressed a higher number of pulmonary leukocytes when compared with unventilated WT mice.

The number of pulmonary leukocytes in ventilated TLR2 KO mice were not different from those in the age matched ventilated WT mice (table 1).

No lipopolysaccharide contamination was detected in our experimental setting and BAL fluid from unventilated mice .

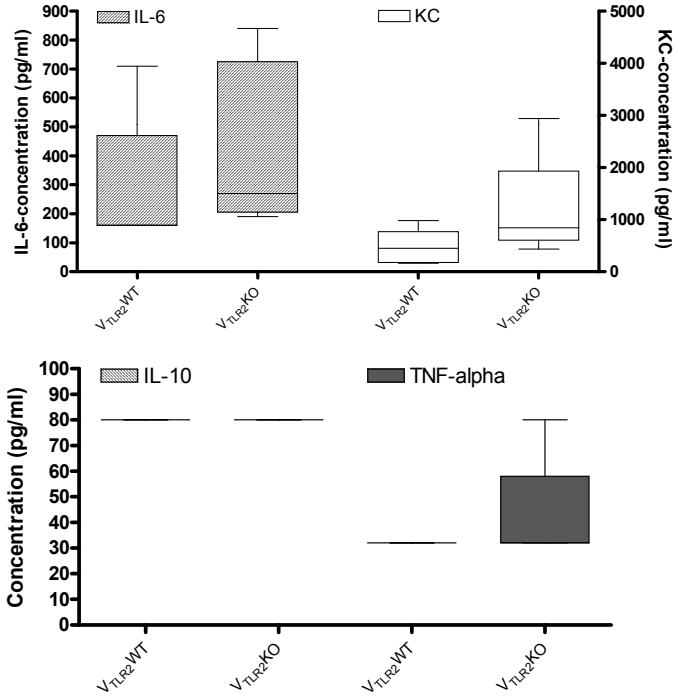

Figure 6: Cytokine levels in plasma.
Levels of interleukin (IL)-6, keratinocyte-derived chemokine (KC), IL-10 and tumor necrosis factor-α in ventilated (V) wild type (WT) and Toll-like receptor (TLR) 2 knock out (KO) mice. Mechanical ventilation in TLR2 KO mice did not result in statistically different cytokine levels in plasma compared with their age matched WT ventilated animals.

Table 1. Leukocyte counts.

Groups	Leukocytes x 10^{-4} / μm^2 Mean (SD)	Delta due to MV	*P* value
C_{TLR4}WT	1.9 (2.6)		
V_{TLR4}WT	8.7 (5.1)	6.8	$P < 0.001$
C_{TLR4}KO	7.2 (5.1)		$P < 0.001$
V_{TLR4}KO	6.1 (4.1)	-1.1	NS
V_{TLR2}WT	3.0 (2.1)		NS
V_{TLR2}KO	4.0 (3.2)	1.0	NS

Values are mean (SD)
MV = mechanical ventilation
NS = not significant
Toll-like receptor (TLR) 4: *P* -values compared to the unventilated WT animals (group C_{TLR4}WT). TLR2: *P* -values compared to the ventilated WT animals (group V_{TLR2}WT).
C = unventilated; KO = knock out; MV = mechanical ventilation; NS = not significant; V = ventilated; WT = wild-type

DISCUSSION

This study is the first to reveal several key findings regarding the role of TLR4 signaling in the development of MV-induced inflammation in healthy mice. Within the time-frame studied, we found that low tidal volume MV resulted in elevated expression of endogenous TLR4 ligands in BAL and enhanced mRNA levels for TLR4 in lung homogenates. In addition, short-term MV resulted in the increase of inflammatory cytokines in the lung and in a systemic inflammatory response in plasma. MV-induced inflammation appeared at least partially TLR4 dependent.

Mechanical ventilation-induced inflammatory response:

The present study confirms previous findings regarding changes in cytokine profile, induced by MV, in lungs and plasma of healthy mice.[24] Little has been published about the effects of low tidal volume MV on cytokines in lungs and plasma of healthy animals. In accord with our results are an increase of IL-1β mRNA expression in lung tissue,[28] an increase of TNF-α concentration in plasma [29] and the lack of response of IL-6 and TNF-α in lung homogenates,[30;31] following low tidal volume MV. The finding of an increased response of IL-6 in plasma but not in lung homogenate can be a time-dependent effect as shown in a previous study.[24] IL-6 levels peaked in lung homogenates after two hours of MV and decreased thereafter. However, in plasma the highest value was reached after 4 hours. In contrast to our results Dhanireddy et al.[30] found no increase of KC in lung homogenate and plasma following low tidal volume MV and Takenaka et al.[32] did not find an increase of IL-6 and TNF-α in plasma following low tidal volume MV. These different results might be partly explained by differences in ventilator settings and experimental set-up in these studies. The present study demonstrates that TLR4 plays a role in development of the inflammatory response following MV in healthy lungs. MV in TLR4 KO did not increase IL-1β in lung homogenates and increases of KC in lung homogenates were less pronounced compared with WT mice. KC and IL-1β are found to play an early and central role in lung injury induced by hemorrhage.[14;33] IL-1β also is involved in lung injury induced by liver injury.[34] Recently IL-1α rather than IL-1β was identified as a key mediator in sterile inflammation in response to injured cells.[35] Our results demonstrate that both IL-1α and IL-1β are involved in the inflammatory response following MV, but only the increase in IL-1β was found to be TLR4 dependent. KC is a chemoattractant, but also has a direct cytotoxic effect.[36] Jiang et al.[17] found KC to be produced by pulmonary epithelial cells in a TLR dependent manner, however this was in direct response to bleomycin. More recently, functional TLR4 expression was found to be critical in the KC increase following hemorrhage.[14] Our results show that KC production in response to MV in healthy lungs appears to be at least partly TLR4 dependent.

The clinical relevance of the cytokine up-regulation by MV is the resulting pro-inflammatory state. This makes the host more vulnerable to a possible "second hit" (*e.g.*, major surgery).[37] Vice versa, MV itself can be the "second hit" where an already comprised host exists (*e.g.*, MV in the critically ill patient).[38-40] Recently this "two hit" hypothesis was linked to TLR4 reactivity.[41]

Inhibition of TLR4 may be an effective strategy to prevent or reduce MV-induced pulmonary and systemic inflammation.

Role for leukocytes:

The activation and attraction of leukocytes is a very important feature in VILI.[42] Leukocytes are thought to be a principal culprit in sterile inflammation causing tissue damage.[35] KC is a major attractant for leukocytes.[43] In a previous study,[24] we have shown that 4 hours of MV in healthy mice preserves alveolar integrity but induces a pulmonary leukocyte influx following the increase of KC in the lung. In the present study this pulmonary leukocyte influx is confirmed, however in TLR4 KO mice MV did not affect the number of pulmonary leukocytes. Recently Frink et al.[14] published a hemorrhage model in which the pulmonary leukocyte influx following hemorrhage was also found to be TLR4 dependent.

In the present study the number of pulmonary leucocytes was significantly higher in unventilated TLR4 KO mice compared with unventilated WT mice. TLR4 KO mice also showed increased lung homogenate levels of KC and IL-1β compared with WT mice at baseline. Frink et al.[14] did not find differences in leukocyte content before hemorrhage, however in that study TLR4 mutant mice were used, which have a different background (C3H/HeJ). Since in these mice the defect is not complete, partial responses of TLR4 could be preserved. In the present study TLR4 KO mice were used. The absence of TLR4 increases their sensitivity to infections.[44] However prior to the experiments these mice did not exhibit clinical signs of discomfort or chronic infection (i.e. abnormal behavior, weight loss). The higher number of pulmonary leukocytes in unventilated TLR4 KO mice is most likely the result of the increased lung homogenate levels of KC.

Role for endogenous ligands:

Recently, a number of studies suggested that endogenous ligands exists that can bind to TLR4 and induce an inflammatory response.[18-23;45] By using HEK293-TLR4 reporter cells, which produce IL-8 in the presence of TLR4 agonists, we sought for endogenous TLR4 ligands in BAL fluid sampled from unventilated and ventilated WT mice. The IL-8 response in BAL fluid from ventilated mice was remarkably enhanced compared with unventilated mice and the addition of a highly specific TLR4 antagonist decreased the induction of IL-8, indicating the presence of endogenous TLR4 ligands in ventilated mice BAL fluid. HEK293-TLR4 cells incubated with BAL fluid from unventilated mice also induced a IL-8 response. This interesting observation could suggest that endogenous ligands for TLR4 are present in the normal (unventilated) lung and released in the air spaces. This might be explained by direct lung injury, induced by performing the BAL procedure as lung lavage is potentially injurious, especially when large volumes are used.[46] We used small volumes for our BAL procedure, but nevertheless cannot exclude that this induces some degree of lung injury and subsequent release of endogenous ligands. The fact that the IL-8 response could only partly be prevented by co-incubation with a TLR4 antagonist

suggests that other non-TLR4 mediated factors play a role. We found that the HEK293-TLR4 cells can also respond to recombinant mouse TNF-α in vitro (personal communication Joosten L.A. and Netea M.G., august 2007). Since small concentrations of TNF-α can be present in BAL fluid of healthy mice,[47] this most likely caused the IL-8 response in the unventilated mice. Most importantly, the IL-8 response was much higher in BAL fluid from ventilated WT mice compared with unventilated WT mice and co-incubation with a TLR4 antagonist prevented this response. This indicates elevated concentrations of endogenous TLR4 ligands following MV.

In a non-infectious lung injury model, Jiang et al.[17] found hyaluronan, which is released from the extracellular matrix, to act as an endogenous ligand and initiate an inflammatory response in a TLR4 dependent manner. Hillman et al.[48] showed that heat shock protein-70, serum amyloid A-3 and TLR2 and TLR4 mRNA were increased in BAL fluid after brief, large tidal volume ventilation in fetal sheep. Since the identification of specific endogenous ligands for TLR4 in healthy lungs after MV was beyond the scope of the present study, further investigation is needed.

The present data suggest that TLR2 does not play a role in the MV induced inflammatory response following short-term MV. Although the relative expression of TLR2 in wild type mice was upregulated, the ventilated TLR2 KO mice did not show differences in cytokine levels compared with age matched ventilated wild type mice after 4 hours of MV. This is in line with preliminary findings in TRIF-deficient mice in which the inflammatory response to MV was clearly suppressed (manuscript in preparation). Since TLR2 signaling is MyD88 dependent and TLR4 is MyD88 or TRIF dependent,[49] it is likely that TLR4-TRIF pathway is the dominant pathway for the inflammatory response to MV. However further studies are needed to establish the role of TLR2 in MV induced inflammation, because we cannot exclude that TLR2 plays a role in the inflammatory response beyond 4 hours of MV.

Factors other than MV, possibly affecting TLR4 and TLR2 signaling were carefully avoided. Contamination with lipopolysaccharide is suggested to be a confounding factor in many studies.[50] We therefore excluded lipopolysaccharide contamination during the experiments. The possibility of triggering an inflammatory response by invasive procedures (i.e. insertion of an intraarterial line) and subsequent bacterial contamination,[37] was eliminated by performing experiments in non-invasively monitored animals. Previously cardiorespiratory stability in invasively monitored animals has been documented.[24] The possible immune modulating effects of anesthetics have been studied extensively.[51] Ketamine, for instance, is known to have an inhibitory effect on lipopolysaccharide-induced cytokine production,[52-55] possibly by suppressing TLR4 expression.[56] Recently, it has been demonstrated that ketamine alone (without lipopolysaccharide) also attenuated cytokine production in humans in the direct postoperative period after elective abdominal surgery.[57] In the present study all animals received ketamine. Ideally, an additional control group of spontaneously breathing animals under ketamine, medetomidine and atropine anesthesia is needed. However, this will result in hypoventilation with severe respiratory acidosis and hemodynamic instability.

Interestingly, a Phase III trial is conducted to investigate the effects of TLR4 inhibition in patients with severe sepsis.[1] The current study supports a role for TLR4 in the inflammatory reaction following MV in healthy lungs. Increasing the understanding of the innate immune response to MV and the contribution of MV to the "multiple hit" concept may lead to future treatment advances in VILI, in which TLR4 may serve as a therapeutic target.

1 http://www.clinicaltrials.gov last accessed: May 26th, 2008

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Chapter 4

Mechanical ventilation induces a Toll/ interleukin-1 receptor domain-containing adapter inducing interferon-beta dependent inflammatory response in healthy mice.

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ABSTRACT

Background:

Mechanical ventilation (MV) can induce lung injury. Pro-inflammatory cytokines have been shown to play an important role in the development of ventilator-induced lung injury. Previously, the authors have shown a role for toll like receptor 4 signaling. The present study aims to investigate the role of Toll/interleukin-1 receptor domain-containing adapter inducing interferon-β (TRIF), a protein downstream of toll like receptors, in the development of the inflammatory response following MV in healthy mice.

Methods:

Wild type C57BL6, and TRIF mutant mice were mechanically ventilated for 4 hours. Lung tissue and plasma was used to investigate changes in cytokine profile, leukocyte influx and nuclear factor-κB activity. In addition, experiments were performed to assess the role of TRIF in changes in cardiopulmonary physiology following MV.

Results:

MV significantly increased messenger RNA expression of interleukin (IL)-1β in wild type mice, but not in TRIF mutant mice. In lung homogenates, MV increased levels of IL-1α, IL-1β and keratinocyte-derived chemokine in wild type mice. In contrast, in TRIF mutant mice only a minor increase in IL-1β and keratinocyte-derived chemokine was found after MV. NF-κB activity after MV was significantly lower in TRIF mutant mice compared with wild type mice. In plasma, MV increased levels of IL-6 and keratinocyte-derived chemokine. In TRIF mutant mice, no increase of IL-6 was found after MV and the increase in KC appeared less pronounced. TRIF deletion did not affect cardiopulmonary physiology following MV.

Conclusions:

The current study supports a prominent role for TRIF in the development of the local and systemic inflammatory response following MV.

INTRODUCTION

Mechanical ventilation (MV) is a life saving therapy in patients with acute respiratory failure. However, studies have shown that MV can aggravate lung injury and even induce lung injury in the healthy lung.[1] The underlying mechanisms are incompletely understood but a large body of literature indicates that pro-inflammatory cytokines play an important role in the development of ventilator-induced lung injury.[2]

Toll-like receptors (TLR) are increasingly being recognized as key mediators in inflammation because of their capacity to detect various microbes and initiate an immune response.[3] In addition, TLRs have been shown to recognize endogenous ligands released from damaged tissue, the so-called 'danger signals'.[4] TLR4 is found to play a role in acute lung injury.[5] Endogenous ligands activate TLR4, resulting in an inflammatory response, which is associated with lung injury.[5] MV using low tidal volume may limit,[6] but does not prevent pulmonary inflammation.[7] Recently, we have shown that in healthy mice, MV with low tidal volume induces a transient inflammatory response, partly in a TLR4-dependent fashion.[8]

Downstream signaling of TLRs is complex. MyD88 is a universal adaptor protein used by most TLRs.[9;10] In TLR4 a second pathway is involved. This pathway is mediated by Toll/interleukin-1 receptor domain-containing adapter inducing interferon-β, a protein called TRIF.[9;10] Activation of TRIF causes delayed translocation of nuclear factor (NF)-κB into the nucleus, and transcription of proinflammatory genes inducing cytokine production.[11] This TRIF pathway has recently been identified as the key signaling pathway in acid-induced lung injury and in hyperoxia-induced lung injury.[5;12] For instance, in TRIF deleted animals acid-induced impairment in lung function and development of lung edema was less pronounced.[5] Whether TRIF is involved in the inflammatory response following MV is currently unknown. This is of interest, as MV is more clinically relevant than acid- or hyperoxia-induced lung injury.

Accordingly, the aim of the present study was to determine the role of TRIF in the development of the inflammatory response and lung function impairment following MV in healthy mice. We hypothesized that MV-induced inflammation involves a TRIF dependent pathway. To test this hypothesis wild type and TRIF mutant mice were mechanically ventilated for 4 hours.

MATERIAL AND METHODS

All experiments were approved by the Regional Animal Ethics Committee (Nijmegen, The Netherlands) and performed under the guidelines of the Dutch Council for Animal Care and the National Institutes of Health.

Animals:

In order to test the role of TRIF in our experimental model, studies were conducted using TRIF mutant mice (C57BL6 background; 10-12 weeks; 25 ± 4 g; N=31). Age matched wild type (WT) mice (C57BL6 background; 26 ± 3 g; N=31) were used as controls. TRIF mutant mice were a kind gift from professor B. Beutler (Department of Immunology, The Scripps Research Institute, La Jolla, CA), who identified and cloned the TRIF gene (called Lps2).[13] These TRIF mutant mice have a distal frameshift error in the Lps2 gene, which has an equivalent gene in humans.[13] WT mice were purchased from Charles River (Sulzfeld, Germany).

Mechanical ventilation in mice:

Mice were anesthetized with an intraperitoneal injection of a combination of ketamine, medetomidine and atropine as described previously.[7] Animals were orally intubated, mechanically ventilated (*MiniVent®*, Hugo Sachs Elektronik-Harvard apparatus, March-Hugstetten, Germany) for 4 hours and killed immediately thereafter. The following settings were used during controlled mechanical ventilation: tidal volume 8 ml/kg body weight and frequency 150 / min, which is well within the range of measured tidal volume and respiratory rate during spontaneous ventilation in C57BL6 mice.[14] All animals received 4 cm H_2O positive end-expiratory pressure and fraction of inspired oxygen was set to 0.4. Rectal temperature was monitored continuously and maintained between 36.0°C and 37.5°C using a heating pad.

Experimental design:

The *first* set of experiments was performed to investigate the role of TRIF in MV-induced changes in cytokine profile, leukocyte influx and NF-κB activity. Blood and lungs were harvested after 4 hours of MV in WT mice (group V-WT, n = 8) and TRIF mutant mice (group V-TRIF, n = 8) or immediately after induction of anesthesia in WT (group C-WT, n = 8) and TRIF mutant mice (group C-TRIF, n = 8)

The *second* set of experiments was designed to assess changes in cardiopulmonary physiology. Continuous intraarterial carotid blood pressure was measured in mechanically ventilated WT mice (group V-WT, n = 15) and TRIF mutant mice (group V-TRIF, n = 15). Arterial blood gas analysis (iSTAT, Abbott, Birmingham, United Kingdom) was performed after 4 hours of MV. We did not include these animals for the cytokine or histopathologic analysis to avoid possible interference with cytokine response resulting from instrumentation (i.e. insertion of arterial catheter) and subsequent bacterial contamination. One TRIF mutant mice died during instrumentation.

Lipopolysaccharide was measured in experimental circuit by Limulus Amebocyte Lysate testing (Cambrex Bio Science, Walkersville, MD; detection limit: 0.06 IU/ml) to rule out contamination with lipopolysaccharide in our experimental setting. Indeed, no lipopolysaccharide could be detected in air, tubing or ventilator (data not shown).

Tissue harvesting:

Blood was collected by exsanguination, centrifuged at 14,000 rpm (13,000 g) (Eppendorf 5415 C, Nethler-Hinz GmbH, Hamburg, Germany) for 2 min and plasma was stored at -80°C for later biochemical analysis. Immediately after exsanguination, heart and lungs were carefully removed *en block* via midline sternotomy. The right upper and lower lobes were snap frozen in liquid nitrogen and stored at -80°C. The right middle lobe was fixed for light microscopy as described previously.[7] The left lung was homogenized for the measurement of cytokines.

Biochemical analysis:

Tumor necrosis factor-α, Interleukin (IL)-6, IL-10, and keratinocyte-derived chemokine (KC) in the homogenized left lung and in plasma were analyzed by enzyme-linked-immunosorbent assay (ELISA) (for Tumor necrosis factor-α, IL-6 and IL10; CytoSet, BioSource, CA; for KC; ELISA-Kit, R&D Systems, MN). Because of insufficient amount of plasma, IL-1α and IL-1β could only be assessed in lung homogenate using specific radio-immuno-assays, as described previously.[15] Lower detection limits: IL-1α and IL-1β: 40 pg/ml; Tumor necrosis factor-α: 32 pg/ml; IL-6: 160 pg/ml; IL-10: 80 pg/ml and KC: 160 pg/ml.

NF-κB's DNA-binding activity was determined by electrophoretic mobility shift assay. Nuclear proteins for electrophoretic mobility shift assay were isolated from liquid nitrogen frozen lungs. Lung tissue (20 mg) was homogenized in 5ml ice-cold buffer (HEPES 10mM, 1.5mM $MgCl_2$, 10mM KCl and 0.6% Nonidet-P40, 0.5 mM dithiothreitol and 0.2 mM phenylmethyl-sulphonylfluoride (Sigma-Aldrich, Zwijndrecht, The Netherlands) and centrifuged for 30s at 350 g (4°C). The supernatant was then incubated on ice for 5 min and centrifuged for 5 min at 6,000 g (4°C). The pellet was resuspended in 200µl buffer (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCL and 1.2 M sucrose, 0.5 mM dithiothreitol and 0.2 mM phenylmethylsulphonylfluoride (Sigma-Aldrich)) and centrifuged for 30 min at 13,000 g (4°C). Then the pellet was resuspended in 66 µl buffer (HEPES 20mM, 1.5mM $MgCl_2$, 0.2 mM EDTA, 420 mM NaCl, 25% glycerol, 0.5mM dithiothreitol, 0.2mM phenylmethylsulphonylfluoride, 2.0 mM benzamidine and 5.0 µg/ml leupeptine (Sigma-Aldrich), incubated on ice for 20 min and centrifuged for 2 min at 6,000 g (4°C). The supernatants were used as nuclear extracts. Protein concentrations in these extracts were determined by using the Bio-Rad protein assay (Bio-Rad, Veenendaal, The Netherlands).

Double stranded oligonucleotides containing an NF-κB consensus binding site (5'-AGTT-GAGGGGACTTTCCCAGGC-3') were radiolabeled with 32[P]-adenosine triphosphate using T4 polynucleotide kinase (Promega, Madison, WI). Labeled NF-κB oligonucleotides were mixed with nuclear extracts (10 µg) and incubated at room temperature for 20 min. Then, these samples were loaded on a 4% polyacrylamide gel. After electrophoresis for 45 min, the gel was dried and exposed for 24 hours to an X-ray film. The bands on the film were quantified using optical densitometry software (GeneTools, Syngene, Cambridge, UK).

mRNA analysis of lung homogenates:

For Polymerase Chain Reaction (PCR) analysis of mRNA, the right upper and lower lobe were homogenized with a micro-dismembrator II (Braun, Melsungen, Germany). Total RNA was extracted in 1 ml TRIzol reagent. Subsequently 200 µL chloroform and 500 µl 2-propanol (Merck, Darmstadt, Germany) were used to separate the RNA from DNA and proteins. After a wash step with 75% ethanol (Merck, Darmstadt, Germany) the dry RNA was dissolved in 30 µL of water. To obtain double strain cDNA, DNase treated total RNA 1 µg with oligo dT primers (0.01 µg/ml) was reverse transcribed in a RT-PCR with a total volume of 20 µl. Subsequently, quantitative PCR was performed using ABI/PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). PCR's of glyceraldehyde-3-phosphate dehydrogenase, IL-1β were performed with Sybr Green PCR Master Mix (Applied Biosystems, Foster City, CA), 5 µL 1/20 diluted cDNA and primers in a final concentration of 300 nmol/L in a total volume of 25 µL. The primers were developed using Primer Express® software (Applied Biosystems, Foster City, CA). Quantification of the PCR signals of each sample was performed by comparing the cycle threshold values (Ct), in duplicate, of the gene of interest with the Ct values of the glyceraldehyde-3-phosphate dehydrogenase housekeeping gene. IL-1β messenger RNA expression was expressed as relative expression to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase. All primers were validated according to the protocol and standard curves were all within the tolerable range.

Leukocytes:

The right middle lung lobe was fixed in 4% buffered formalin solution overnight at room temperature, dehydrated and embedded in paraplast (Amstelstad, Amsterdam, The Netherlands). Sections of 4 µm-thicknesses were used. The enzyme activity of leukocytes was visualized by enzyme histochemistry using chloracetatesterase staining (Leder staining). Leukocytes were counted manually (20 fields per mouse), and after automated correction for air/tissue ratio, the number of leukocytes/μm^2 was calculated.

Statistical Analysis:

Data are expressed as mean (SD) when distributed normally (leukocyte counts, relative mRNA expression) and expressed as Box (median, 25th, 75 percentile) and Whiskers (range) otherwise (cytokine concentrations). Statistical analysis was performed with SAS (SAS Institute Inc. Cary NC, USA, version 8.02) statistical procedures. Since cytokine concentrations are not normally distributed, Kruskal Wallis procedures were used, with post hoc comparisons of subgroups (Duncan). For the analysis of normally distributed data (leukocyte counts and relative mRNA expression) ANOVA was used with post hoc comparison of group means (Duncan). Two-tailed P-values are reported and the level of significance was set to $P < 0.05$.

RESULTS

Inflammatory response after mechanical ventilation: role for TRIF:

LUNGS: In WT mice, MV increased levels of IL-1α, IL-1β and KC (figure 1), which is in line with previous data from our lab.[7;8] In contrast, in TRIF mutant mice MV elicited only a minor increase in IL-1β and KC. Levels of IL-1α after MV were not different between WT and TRIF mutant mice (figure 1). In addition MV increased the messenger RNA expression of IL-1β in WT mice, but not in TRIF mutant mice following MV, indicating that TRIF affects IL-1β transcription (figure 2). NF-κB activity after MV was significantly lower in TRIF mutant mice compared with WT mice (figure 3).

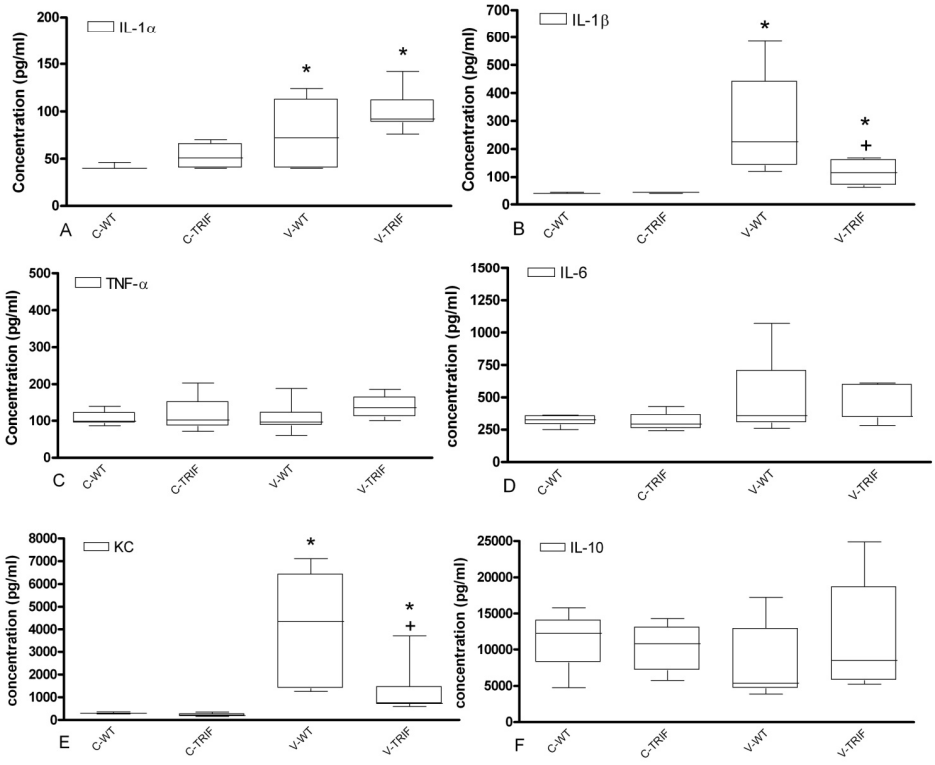

Figure 1: Cytokine levels in lung homogenates.
Levels of interleukin (IL)-1α, IL-1β, tumor necrosis factor-α, IL-6, keratinocyte-derived chemokine (KC), and IL-10 in unventilated (C) and ventilated (V) wild type (WT) and TRIF mutant (TRIF) mice (panels A-F). Mechanical Ventilation in WT mice (group V-WT) increased IL-1α ($P = 0.03$), IL-1β ($P < 0.0001$) and KC ($P < 0.0001$) in lung tissue homogenates when compared with unventilated WT mice (group C-WT). Mechanical ventilation in TRIF mutant mice (group V-TRIF) increased levels of IL-1α ($P = 0.01$), IL-1β ($P = 0.0004$) and KC ($P < 0.0001$) in lung tissue homogenates when compared with unventilated TRIF mutant mice (group C-TRIF). Ventilated TRIF mutant mice (V-TRIF) showed significantly lower levels of IL-1β ($P = 0.01$) and KC ($P = 0.01$) in lung homogenates following mechanical ventilation compared with ventilated WT mice (group V-WT). Data are expressed as Box (median, 25th, 75 percentile) and Whiskers (range). * = $P < 0.05$ compared with unventilated mice. + = $P < 0.05$ compared with ventilated WT mice (V-WT). - = lower detection limit.

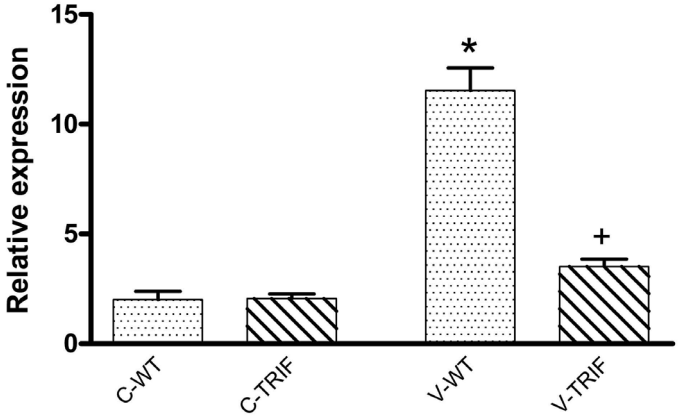

Figure 2: Pro-IL-1β levels in lung tissue.
Pro-IL-1β messenger RNA levels in unventilated (C) and ventilated (V) wild type (WT) and TRIF mutant (TRIF) mice. Mechanical Ventilation in WT mice (group V-WT) increased pro-IL-1β mRNA ($P < 0.0001$) in lung tissue when compared with unventilated WT mice (group C-WT). In TRIF mutant mice no increase in mRNA expression of IL-1β was found following mechanical ventilation. Ventilated TRIF mutant mice (V-TRIF) showed significantly lower levels of pro-IL-1β ($P < 0.0001$) in lung tissue following mechanical ventilation compared with ventilated WT mice (group V-WT). Data are expressed as mean (SD) .
* = $P < 0.05$ compared with unventilated mice. + = $P < 0.05$ compared with ventilated WT mice (V-WT).

MV resulted in a pulmonary leukocyte influx in both WT and TRIF mutant mice (figure 4).
PLASMA: In WT mice, MV increased levels of IL-6 and KC in plasma (figure 5). In TRIF mutant mice, no increase of IL-6 was found after MV and the increase in KC appeared less pronounced.

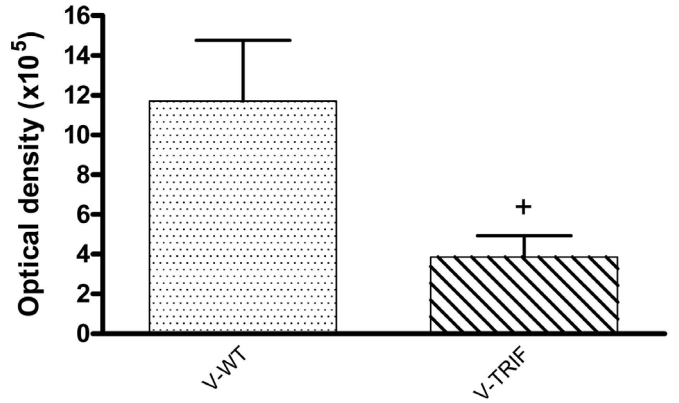

Figure 3: NFkB activity in lung tissue.
NFκB activity in lung tissue of ventilated (V) wild type (WT) and TRIF mutant (TRIF) mice. In ventilated TRIF mutant mice (group V-TRIF) NFκB activity was significantly lower ($P < 0.0001$) compared with ventilated WT mice (group V-WT). Data are expressed as optical densities from electrophoretic mobility shift assay analysis. + = $P < 0.05$ compared with ventilated WT mice (V-WT).

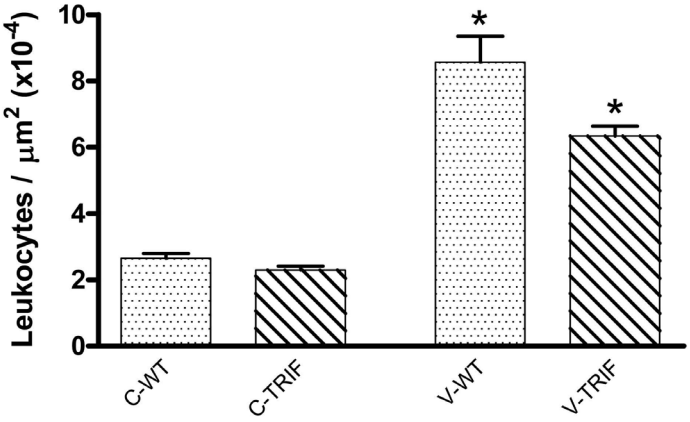

Figure 4: Leukocyte counts.
Mechanical ventilation resulted in a pulmonary leukocyte influx in WT mice (group V-WT) ($P < 0.0001$) compared with unventilated WT mice (group C-WT). In TRIF mutant mice (group V-TRIF) mechanical ventilation also increased pulmonary leukocyte counts ($P < 0.0001$) compared with unventilated WT mice (group C-WT). The pulmonary leukocyte number in ventilated TRIF mutant mice appeared lower compared with ventilated WT mice (group V-WT), however this did not reach statistical significance ($P = 0.07$).

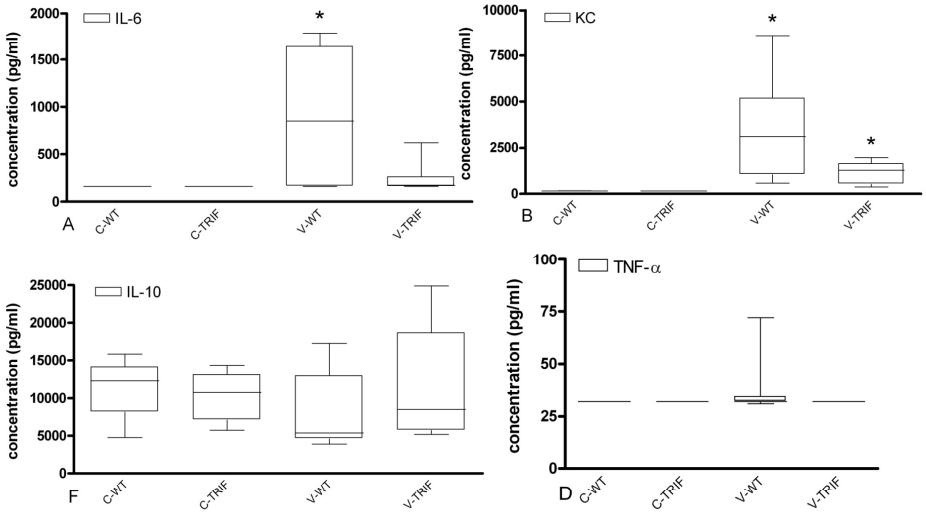

Figure 5: Cytokine levels in plasma.
Levels of interleukin (IL)-6, keratinocyte-derived chemokine (KC), IL-10 and tumor necrosis factor-α in unventilated (C) and ventilated (V) wild type (WT) and TRIF mutant (TRIF) mice (panels A-D). Mechanical ventilation in WT mice (group V-WT) increased IL-6 ($P = 0.0009$), and KC ($p < 0.0001$) in plasma when compared with unventilated WT mice (group C-WT). In TRIF mutant mice (group V-TRIF) no increase in IL-6 was found following mechanical ventilation when compared with the unventilated TRIF mutant mice (group C-TRIF). Mechanical ventilation in TRIF mutant mice (group V-TRIF) did increase levels of KC ($P < 0.0001$) when compared with unventilated TRIF mutant mice (group C-TRIF). This was not statistically different from ventilated TRIF mutant mice (group V-TRIF). Data are expressed as Box (median, 25th, 75 percentile) and Whiskers (range). * = $P < 0.05$ compared with unventilated mice. - = lower detection limit.

Cardiopulmonary physiology:

The animals with an intraarterial canula exhibited stable hemodynamic variables throughout the experiments. Mean arterial pressure was within normal limits and remained above 65 mmHg in all animals, which was in line with previous data from our lab.[7] Blood gas analysis showed pH: 7.30 ± 0.07 in WT mice and 7.32 ± 0.08 in TRIF mutant mice; arterial oxygen tension: 146 ± 23 mmHg in WT mice and 157 ± 20 mmHg in TRIF mutant mice; arterial carbon dioxide tension: 41 ± 6 mmHg in WT mice and 39 ± 5 mmHg in TRIF mutant mice; bicarbonate: 17.4 ± 3 mmol/l in WT mice and 19.1 ± 3.1 mmol/l in TRIF mutant mice. PaO_2 / FiO_2 ratio's and alveolar-arterial oxygen gradients were not different between WT and TRIF mutant mice after MV.

DISCUSSION

The present study confirms earlier observations from our laboratory,[7;8] and others,[1;6;16] that MV using clinical relevant ventilator settings results in a pulmonary and systemic inflammatory response. The present study extends these findings by showing that TRIF deficiency attenuates this inflammatory response following MV, by reducing NF-κB activation. TRIF deletion prevented pulmonary pro-IL-1β increase and systemic IL-6 increase after 4 hours of MV. Also, pulmonary levels of IL-1β and KC were significantly lower in TRIF deleted mice lungs compared with WT lungs after MV.

The TRIF pathway is a downstream pathway of TLR4 and TLR3,[9;10] that can cause delayed NF-κB activation.[17] Recently, we have shown the involvement of TLR4 in MV-induced inflammation.[8] The present study extends these findings by showing the involvement of TRIF signaling. We found that the MV-induced increase of KC and IL-1β was TRIF dependent. Since TRIF is also involved in the downstream signaling of TLR3 we cannot exclude the involvement of TLR3 in our model. However the results presented here closely resemble the results from our previous TLR4 experiment.[8] Therefore it is likely that in downstream signaling of TLR4 the TRIF pathway is involved in the inflammatory response following MV. Subsequent studies using TLR3 deleted mice are needed to confirm the importance of TLR4 in MV-induced inflammation.

Potential relevance of findings:

Several studies have pointed out the involvement of the analyzed cytokines in lung injury. IL-1β has been shown to be among the most biologically active cytokines in the lungs,[18;19] and therefore is proposed to play an important role in the pathogenesis of lung injury.[20] In clinical studies, high tidal volume MV results in persistently high plasma levels of IL-1β, which is associated with distal organ failure and mortality.[2;21] In the present study, TRIF deletion prevented IL-1β increase at messenger RNA level and attenuated the increase of IL-1β at protein levels indicating TRIF involvement at the level of transcription. This is interesting as it indicates major

involvement of the TRIF pathway and may indicate only minor or no influence of the MyD88 pathway. However this hypothesis needs further investigation.

KC is a chemoattractant, but also has a direct cytotoxic effect.[22] Jiang et al.[23] found KC to be produced by pulmonary epithelial cells in a TLR4 dependent manner in direct response to bleomycin. More recently, functional TLR4 expression was found to be critical in the KC increase following hemorrhage.[24] The present study shows that the increase of KC following MV is at least partly TRIF dependent.

Our data show that blocking TRIF dependent pathway prevents the increase in plasma IL-6 after MV. Several studies suggest that Il-6 plays a role in the development of distal organ failure in ventilator-induced lung injury.[2;25] Accordingly, TRIF modulation may attenuate distal organ failure induced by MV.

It should be noticed that in our model no evidence of either pulmonary dysfunction or distant organ failure was found, despite the development of an inflammatory response. Apparently, the trigger induced by the ventilator is relatively mild and the lung is able to cope with the MV-induced inflammatory reaction. This has been demonstrated before in a study from our laboratory,[7] and by a clinical study showing that 2 hours of MV in healthy children resulted in enhanced cytokine concentrations without clinical signs of pulmonary dysfunction.[16] In acid-induced lung injury TRIF deletion indeed diminished impairments in lung function.[5] Therefore, the inflammatory response following MV in healthy lungs may be too subtle to identify changes in cardiopulmonary physiology. However, the inflammatory response following MV is clinically relevant as this forms the basis for the two-hit hypothesis proposing that injury (*e.g.*, the critically ill patient) primes the immune system ("first hit") for a lethal inflammatory reaction to a later, otherwise nonlethal, secondary insult (second hit), namely MV.[26-28] This enhanced host response can lead to distal organ failure and is previously linked to TLR4 reactivity,[29] which is interesting as we have shown that TLR4 is involved in the inflammatory response following MV in healthy lungs.[8] Downstream of TLR4 we identified the involvement of TRIF in this response. Therefore, inhibition of TRIF may be an effective strategy to prevent or attenuate MV induced pulmonary and systemic inflammation. Figure 6 shows a schematic overview of the downstream signaling pathways of TLR4.

KC is a major chemoattractant for leukocytes.[30] In a previous study, we have shown that 4 hours of MV in healthy mice induces a TLR4 dependent pulmonary leukocyte influx following the increase of KC in the lung.[7;8] In the present study this pulmonary leukocyte influx is confirmed. In TRIF mutant mice leukocyte influx appeared less pronounced, however this did not reach statistical significance. This might be explained by the fact that in plasma, the increase in KC in TRIF mutant mice also appeared to be reduced, without reaching statistical significance.

Our study has several limitations. First, all studies were performed in mice. It is unknown whether the response to MV in mice is similar to the response in humans. Second, only the effect of 4 hours of MV was tested. In a previous study we have shown that the proinflammatory response is activated within 30 minutes after initiation of MV and intensifies up to 4

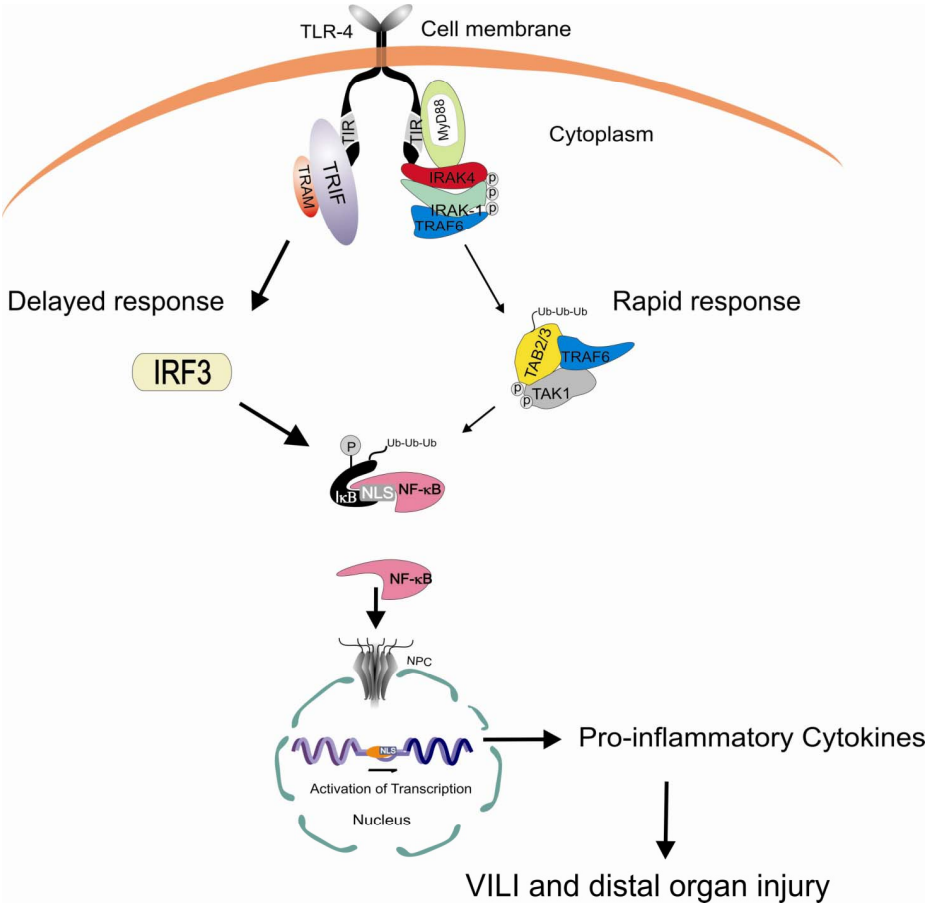

Figure 6: Downstream signaling of Toll-like receptor 4.
Schematic representation of downstream signaling pathways of the Toll-like receptor 4. Two pathways are involved. The rapid MyD88 dependent response and the delayed Toll/interleukin-1 receptor domain-containing adapter inducing interferon-β (TRIF) response also known as the MyD88 independent response. Both signaling routes cause translocation of nuclear factor (NF)-κB into the nucleus, and transcription of proinflammatory genes inducing cytokine production. Our challenging hypothesis is that these cytokines induce a pro-inflammatory response, which can result in ventilator induced lung injury and distal organ injury. It should be noticed that activation of TLR3 may also activate TRIF dependent pathway (not shown in figure).

hours after initiation of MV.[7] Preliminary observations show that the inflammatory response unaltered after 8 hours of MV compared with 4 hours of MV. Third, in the present study we did not evaluate the effect of different ventilatory modes. Interstingly, recent studies indicate that spontaneous ventilatory efforts may improve respiratory function such as diaphragm function, pulmonary gas exchange and hemodynamics following MV.[31;32]

Factors other than MV, possibly affecting TRIF were carefully avoided. Contamination with lipopolysaccharide is suggested to be a confounding factor in many studies.[33] We therefore

excluded lipopolysaccharide contamination during the experiments. The possibility of triggering an inflammatory response by invasive procedures (i.e. insertion of an intraarterial line),[34] was eliminated by performing experiments in non-invasively monitored animals. Previously cardiopulmonary stability in invasively monitored animals has been documented.[7] In the present study mice were slightly acidotic following MV. As hypercapnic acidosis attenuates MV-induced inflammation in healthy mice,[35] this could affect our results. However it is unlikely that the slight metabolic acidosis in the present study significantly affects our data. Indeed, correcting metabolic acidosis does not alter levels of cytokines following MV in healthy mice.[36] The possible immune modulating effects of anesthetics have been studied extensively.[37] Ketamine, for instance, is known to have an inhibitory effect on lipopolysaccharide-induced cytokine production,[38-41] possibly by suppressing TLR4 expression.[42] Recently, it has been demonstrated that ketamine alone (without lipopolysaccharide) also attenuated cytokine production in humans in the direct postoperative period after elective abdominal surgery.[43] In the present study all animals received ketamine. Ideally, an additional control group of spontaneously breathing animals under ketamine, medetomidine and atropine anesthesia is needed. However, this will result in hypoventilation with severe respiratory acidosis and hemodynamic instability.

The current study supports a role for TRIF in the inflammatory reaction following MV in healthy lungs. Increasing the understanding of the innate immune response to MV and the contribution of MV to the "multiple hit" concept may lead to future treatment advances in VILI, in which TRIF may serve as a therapeutic target.

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Chapter 5

Hypercapnic acidosis attenuates the pulmonary innate immune response in ventilated healthy mice.

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ABSTRACT

Background:

Mechanical ventilation (MV) with small tidal volumes reduces the development of Ventilator Induced Lung Injury (VILI) and mortality, but may increase $PaCO_2$. It is not clear whether the beneficial effect of a lung-protective strategy results from reduced ventilation pressures/tidal volumes or is mediated by the effects of hypercapnic acidosis on the inflammatory response involved in the pathogenesis of VILI. To analyze whether hypercapnic acidosis affects lung tissue cytokine levels and leukocyte influx healthy mice were ventilated.

Methods:

Healthy C57BL6 mice (n=40) were mechanically ventilated. Analysis of lung tissue and plasma concentrations of IL-1β, TNF-α, IL-6, IL-10 and keratocyte-derived chemokine (KC) after 2 hours of mechanical ventilation (Vt 8 ml/kg, PEEP 4 cmH_2O) with 0.06% CO_2 (room air), 2% CO_2 or 4% CO_2.

Results:

$PaCO_2$ and pH were within normal range when ventilated with 0.06% CO_2 and significantly changed with 2% and 4% CO_2: (mean±SD) pH 7.23±0.06 and 7.15±0.04, $PaCO_2$ 7.9±1.4 and 10.8±0.7 kPa, respectively (p<0.005). Blood pressure remained within normal limits in all animals. Quantitative microscopic analysis showed a 4.7±3.7 fold increase (p<0.01) in pulmonary leukocyte influx in normocapnic ventilated animals and a significant reduction in leukocyte influx of 57±32% (p<0.01) and 67±22% (p<0.01) when ventilated with 2% and 4% CO_2, respectively. Normocapnic ventilation induced a significant elevation of lung tissue IL-1β (1516±119 ng/ml), TNF-α (344±88 ng/ml), IL-6 (6310±807 ng/ml), IL-10 (995±152 ng/ml) and KC (36966±15294 ng/ml) (all p-values <0.01). Hypercapnic acidosis with 2% resp. 4% CO_2 significantly attenuated this increase with 25±32% and 54±32% (IL-1β, p<0.01); 17±36% and 58±33% (TNF-α, p<0.02); 22±34% and 89±6% (IL-6, p<0.01); 20±31% and 67±17% (IL-10, p<0.01) and 16±44% and 45±30% (KC, p=0.07)

Conclusions:

Hypercapnic acidosis attenuates the mechanical ventilation-induced immune response independent from reduced tidal volumes/pressures and may protect the lung from Ventilator Induced Lung Injury.

INTRODUCTION

Mechanical ventilation (MV) can induce or exacerbate lung injury through a process of barovolutrauma and inflammation, termed *Ventilator Induced Lung Injury (VILI)*. Reducing tidal volumes and peak pressures ("Lung Protective Ventilation") not only results in both lower pulmonary cytokine levels but also decreased lung injury and mortality.[1-3] However, low tidal volume ventilation may result in an increase in $PaCO_2$ ("permissive hypercapnia"). Severe hypercapnic acidosis is usually well tolerated,[4;5] and several clinical studies even suggest a protective effect on various organs, including the ventilated lung.[6-9] A retrospective multi-variate logistic regression analysis of the ARDS network trial suggests that hypercapnic acidosis may be protective in ARDS patients ventilated with high tidal volumes.[10]

Several mechanisms may explain the effect of hypercapnic acidosis on VILI. First, accepting a higher $PaCO_2$ level allows the use of smaller tidal volumes and lower airway pressures, which has beneficial effects on VILI, as shown in several clinical trials.[5-7] Second, hypercapnic acidosis has direct effects on lung perfusion, oxygenation and oxygen delivery. Both experimental and clinical studies have shown that hypercapnic acidosis increases tissue oxygen delivery, by shifting the oxygen dissociation curve to the right and improving V/Q match.[11-13] Third, an increase in $PaCO_2$ may have a direct modulating effect on the inflammatory response. Some of these effects are probably protective, such as inhibition of xanthine oxidase and oxygen radical formation,[14] decreased NF-kB transcriptional activity,[15] and decreased complement activation.[16] Other effects are potentially harmful, e.g. reduced neutrophil burst and superoxide formation,[16] inhibition of NO synthases,[17] and delayed apoptosis.[18;19] The biochemical pathways on a cellular level include altered electrochemical membrane potentials, alterations in micro-tubuli assembly, lowered enzyme activity and altered gene transcription as reviewed by Kregenow.[16]

In the clinical setting it is difficult to establish which of the two variables, reduced tidal volume/pressure or hypercapnic acidosis, accounts for the observed improvement in outcome. We conducted a series of experiments to establish the effect of hypercapnic acidosis on ventilation-induced pulmonary cytokine increase and leukocyte influx by administration of inhaled CO_2, while maintaining identical ventilator settings. We show that hypercapnic acidosis significantly decreases lung leukocyte influx and cytokine release.

MATERIAL AND METHODS

All experiments were approved by the Regional Animal Ethics Committee Nijmegen and performed under the guidelines of the Dutch Council for Animal Care and the National Institutes of Health.

Animals:

C57BL6 mice (n=40, Charles River, Sulzfeld, Germany), 10 - 12 weeks of age, with a weight ranging from 23-28 g were used.

Mechanical ventilation in mice:

Anesthesia was induced and maintained with intraperitoneal administration of a combination of Ketamine, Medetomidine and Atropine (KMA) as reported before.[20] Animals were orally intubated under direct vision with an endotracheal tube (0.82 mm ID, 1.1 mm OD, length 25 mm). Subsequently, animals were connected to the ventilator (*MiniVent®*, Hugo Sachs Elektronik - Harvard apparatus, March-Hugstetten Germany). Tidal volume (Vt) was set at 8 ml/kg, which is comparable with Vt during spontaneous ventilation in C57BL6 mice.[21] All animals were ventilated with 4 cm H_2O PEEP. Studies using broncho-alveolar lavage did not find increased cytokine levels with similar ventilatory settings, however, using tissue homogenate we previously demonstrated increased intracellular and tissue cytokine levels, without evoking histological changes.[20;22-24] To avoid direct oxygen toxicity [25;26] the FiO_2 was set at 0.4. Respiratory rate was set and fixed at 150/min to obtain normocapnia with 0.06% CO_2 (room air). An intra-arterial carotid canula was inserted for continuous blood pressure monitoring and blood gas analysis at the end of the experiment. Throughout the experiment rectal temperature was monitored and maintained between 36.5° and 37.5° C using a heating pad.

Study groups:

In the first set of experiments, animals were randomly divided into 4 groups. Group I (n=6) was not ventilated, immediately sacrificed after induction of anesthesia and served as the control group. Group II (n=6) was ventilated with 0.06 % CO_2. Group III (n=6) was ventilated with 2% CO_2, and Group IV (n = 6) was ventilated with 4% CO_2. Ventilator settings in Group II, III and IV were identical as described above.

In a second set of experiments we used the same protocol to measure pulmonary wet/dry ratios. These animals were ventilated as in group II (n=4), III (n=4) and IV (n=4). Four spontaneously breathing animals served as controls (n=4).

Material harvesting and preparation of lung tissue:

Mechanical ventilation lasted two hours in all groups, after which the mice were sacrificed by exsanguination. Blood was collected from the arterial line, centrifuged at 13,000 g (Eppendorf 5415 C, Nethler-Hinz GmbH, Hamburg) for 2 minutes and the plasma was stored at -80°C. Immediately after exsanguination, the heart and lungs were carefully removed *en block* via midline sternotomy. The right middle lobe was fixed for light microscopy. The remaining lung tissue was homogenized for the determination of cytokine concentrations.

To assess pulmonary edema, wet/dry ratios of both lungs were used; ratios were calculated by measuring lung weight before and after heating for 24 hours in a stove at 40°C.

Light microscopy:

For light microscopy the material was fixed in 4% buffered formalin solution overnight at room temperature, dehydrated and embedded in paraplast (Amstelstad, Amsterdam, The Netherlands). Sections of 4 µm-thickness were used for analysis. The enzyme activity of leukocytes was visualized by enzyme histochemistry using chloracetatesterase staining (Leder staining). Leukocytes were counted manually (20 fields per mouse), and after automated correction for air/tissue ratio, leukocytes/µm^2 were calculated. The pathologist was blinded for the group and ventilation protocol.

Laboratory tests:

Levels of TNF-α, IL-6, IL-10, and Keratocyte-derived chemokine (KC) in lung homogenate were measured using Enzyme-Linked-Immunosorbent Assay (ELISA) (for TNF-α, IL-6 and IL10; CytoSet, BioSource, USA; for KC; ELISA-Kit, R&D, USA). IL-1β was assessed using specific radio-immuno-assays (Nijmegen University, Netherlands), as described previously.[20;27] Lower limit of detection for IL-1β was 40 pg/ml, for TNF-α 32 pg/ml, for IL-6 160 pg/ml, for IL-10 16 pg/ml and for KC 160 pg/ml.

Statistical analysis:

Data are expressed as mean (SD). If cytokine concentrations were below the detection limit, lower limits of detection were used for further calculations.

Comparison between groups was performed using one-way ANOVA. A p-value <0.05 was considered statistically significant. All tests and graphs were constructed using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA.

RESULTS

Cardio respiratory parameters:

As illustrated in table 1, animals ventilated without supplemental CO_2 (Group II) exhibited normal pH, PaO_2 and $PaCO_2$ at the end of experiment, comparable with values in spontaneously breathing mice. Increasing inspiratory CO_2 (group III and group IV) resulted in a significantly elevated $PaCO_2$ and a decrease in pH ($p<0.05$) (table I), whereas base excess did not differ

Table 1: Arterial blood gas analysis.

	pH	*PaO_2*	*$PaCO_2$*	*HCO_3^-*	*BE*
0.06% CO_2	7.36±0.06	28.9±10.4	5.0±0.9	20.5±1.2	-4.4±1.5
2% CO_2	7.23±0.06*	27.2±4.9	7.9±1.4*	21.4±0.9	-4.5±1.2
4% CO_2	7.15±0.04*	28.9±1.3	10.8±0.7*	22.7±1.8	-3.8±2.8

PaO_2, $PaCO_2$ in kPa;
* $p<0.005$ 2, 4% CO_2 vs. 0.06% CO_2

between groups. In all animals, invasive arterial blood pressure (IABP) remained within normal limits throughout the experiment and did not differ between groups (table II).

Table 2: Arterial blood pressure.

	T=0.5 hr	*T=1 hr*	*T=1.5 hr*	*T= 2 hr*
0.06% CO_2	95.6±15.7	95.3±15.9	78.6±8.1	80.1±10.2
2% CO_2	92.1±14.3	78.6±6.9	76.2±7.9	74.3±2.4
4% CO_2	92.5±5.1	85±7.2	79.25±3.3	81.5±8.1

Arterial blood pressure in mmHg

Light microscopy:

Mechanical ventilation significantly increased lung leukocyte counts in animals ventilated with 0.06% CO_2 from 207±133 / mm^2 to 971±496 / mm^2 (group I vs. group II, $p<0.01$). MV with 2% and 4% CO_2 (group III and IV) attenuated the increase in leukocyte influx with 57±32% and 67±22% ($p<0.01$), respectively (figure 1). No alveolar tissue disruption or other structural damage was observed and all detected leukocytes remained within the lung interstitium.

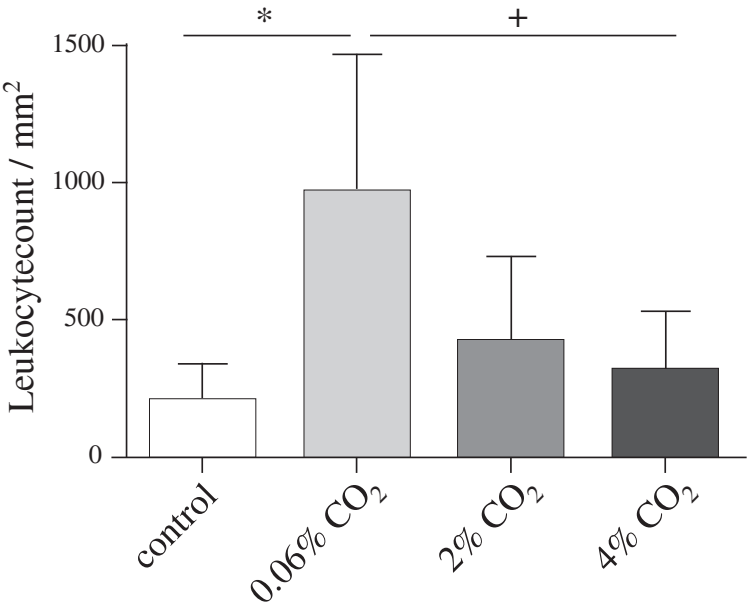

Fig 1: Leukocyte counts.
Effect of two hours normo- and hypercapnic mechanical ventilation on leukocyte counts in lung tissue, compared with unventilated control animals. *P<0.05; + p<0.05 0.06 CO_2 vs 2% CO_2 vs 4% CO_2.

Wet/dry ratio:

There were no significant differences in wet/dry ratios between the four groups (data not shown, all p>0.2).

Cytokine concentrations:

IL-1β, TNF-α, IL-6, IL-10 and KC lung tissue levels were significantly increased after 2 hours of normocapnic MV: IL-1β from 42±4 to 1516±119 pg/ml; TNF-α from 42±18 to 344±88 pg/ml; IL-6 from 264±130 to 6310±807 pg/ml; IL-10 from 233±124 to 995±152 pg/ml; KC from 384±217 to 36967±15294 pg/ml (all p-values<0.01). In group III (2% CO_2) and group IV (4% CO_2), hypercapnic MV significantly attenuated this increase in IL-1β with 25±32% and 54±32% (p=0.006); TNF-α 17±36% and 58±33% (p=0.019); IL-6 22±34% and 89±6% (p=0.001); IL-10 20±31% and 67±17% (p=0.005) and KC 16±44% and 45±30% (p=0.07) (Fig 2).

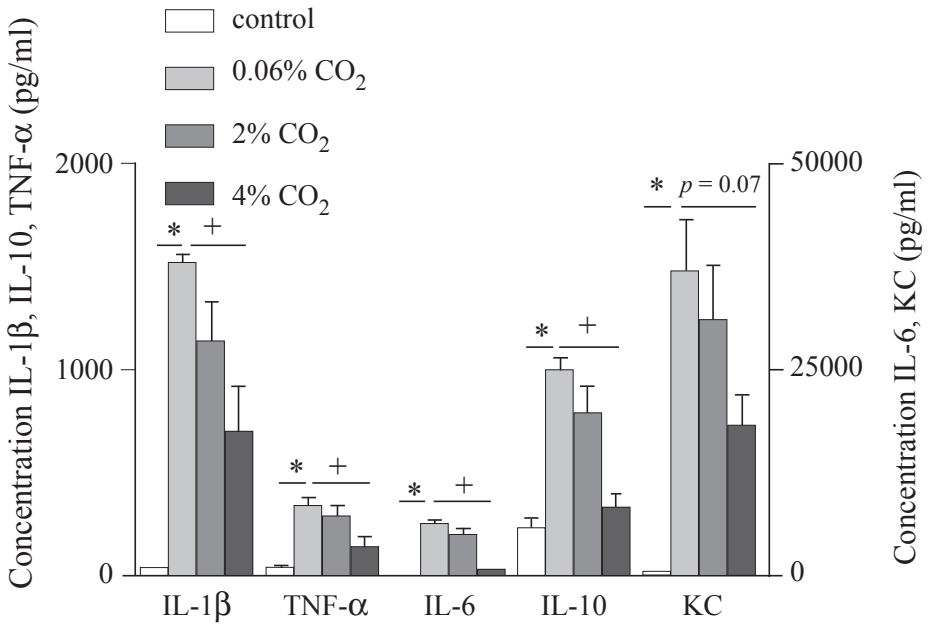

Fig 2: Cytokine levels in lung homogenates
Effect of two hours normo- and hypercapnic mechanical ventilation on ventilation-induced lung tissue cytokine release using identical ventilator settings.
* P<0.05 vs. control; + p<0.05 0.06% CO_2 vs 2% CO_2 vs 4% CO_2.

DISCUSSION

In the present study we demonstrate that the ventilation-induced increase in leukocyte influx and pulmonary cytokines, a hallmark of VILI, is significantly attenuated in hypercapnic-acidotic animals ventilated with the same tidal volumes. Hypercapnic acidosis may represent an

important pathway to protect the lung against VILI. To our knowledge, this study demonstrates for the first time the attenuating effect of hypercapnic acidosis on cytokine levels *in vivo*. As all other cardiorespiratory and ventilatory parameters remained identical between ventilated groups, we assume that the observed differences are entirely attributable to differences in hypercapnic acidosis.

Our *in vivo* findings are in concordance with several *in vitro* observations. Lang et al.[28] showed that hypercapnia attenuates TNF-α production in lipopolysaccharide (LPS)-stimulated rabbit and rat alveolar macrophages *in vitro*. In human LPS-stimulated polymorphonuclear neutrophilis and alveolar macrophages hypercapnic acidosis decreased the production of IL-8.[15;29] IL-8 is a potent chemoattractant and comparable with KC in animals. We showed an attenuated increase in KC during ventilation with hypercapnic acidosis. We previously demonstrated that mice ventilated for 30 minutes with identical ventilator settings had increased KC levels in lung homogenate prior to leukocyte influx, and that leukocyte depleted mice demonstrated a significant mechanical ventilation-induced increase in KC but hardly any increase in IL-1β, TNF-α, IL-6 and IL-10.[20] The attenuated KC increase during hypercapnic acidosis may explain the decrease in leukocyte infiltration as observed by us and by others studying non-ventilation related primary lung injury (pulmonary LPS installation) and secondary lung injury (ischemia reperfusion) *in vivo*.[30-32]

Once cytokine up-regulation and leukocyte chemotaxis are triggered by mechanical ventilation, further propagation of the inflammatory response by release of cytokines from leukocytes through positive feedback occurs. Experimental administration of a IL-1β receptor antagonist and TNF-α monoclonal antibodies appear to attenuate the development of VILI, demonstrating the involvement of cytokines in its pathogenesis.[33-35] Moreover, several clinical studies clearly demonstrated the relation between cytokine levels and morbidity.[36;37] Another cytokine-propagating mechanism affected by hypercapnic acidosis may be decreased NF-κB activation. NF-κB appears to be stimulated by both IL-1β and TNF-α and regulates gene transcription responsible for IL-6 and KC synthesis.[38] Hypercapnic acidosis inhibits NF-κB translocation into the nucleus by suppressing degradation of IκB-α, a regulatory protein that binds the cytoplasmatic inactive NF-κB.[15;39] This pathway may offer an explanation for the reported attenuation of NF-κB mediated IL-6 and KC increase in hypercapnic acidosis. Further experiments are necessary to elucidate what the effects of hypercapnic acidosis during MV are on the cellular mechanisms proposed above.

From our experiment it remains unclear whether it is the hypercapnic acidosis or the resultant acidosis that is responsible for the observed beneficial effects. In *ex vivo* lung injury experiments hypercapnic acidosis appears to preserve alveolo-capillary integrity more than metabolic acidosis.[40] Also, synergistic effects between $PaCO_2$ and pH may occur, as buffer therapy for hypercapnic acidosis reduces its protective effects.[40] A limitation of our study model is that the ventilator setting, although clinically relevant, results in functional dysregulation with

elevated cellular and tissue cytokine levels, but not in histological lung injury.[20] Therefore, our study does not enable us to conclude that hypercapnic acidosis also better preserves pulmonary integrity. However, the conclusions regarding the pathophysiological importance of the increased cytokine levels still hold. As ventilation duration in our study was limited to two hours, the observed beneficial effects can not directly be extrapolated to outcome when ventilated for longer periods. Also, whether hypercapnic acidosis also attenuates the cytokine response in an injured lung with an otherwise activated innate immune response as encountered in the clinical setting, needs further study.

In conclusion, hypercapnic acidosis by itself directly attenuates pulmonary leukocyte influx and cytokine release during mechanical ventilation, an effect that is independent from reduced tidal volume and pulmonary pressures. The observation that hypercapnic acidosis directly modulates the mechanical ventilation-induced innate immune response in the lung suggests that this pathway may account for the beneficial effects of hypercapnic acidosis in critically ill ventilated patients. The net effect of hypercapnic acidosis on pulmonary and other end organ function in the clinical setting remains to be determined.

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Chapter 6

Isoflurane attenuates pulmonary IL-1β and systemic TNF-α following mechanical ventilation in healthy mice.

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ABSTRACT

Background:

Mechanical Ventilation (MV) induces an inflammatory response in healthy lungs. The resulting pro-inflammatory state is a risk factor for ventilator-induced lung injury and peripheral organ dysfunction. Isoflurane is known to have protective immunological effects on different organ systems. We tested the hypothesis that the MV-induced inflammatory response in healthy lungs is reduced by isoflurane.

Methods:

Healthy C57BL6 mice (n=34) were mechanically ventilated (tidal volume, 8 ml/kg; positive end-expiratory pressure, 4 cm H_2O; fraction of inspired oxygen, 0.4) for 4 hours under general anesthesia using a mix of ketamine, medetomidine and atropine (KMA). Animals were divided into 4 groups: 1] Unventilated control group. 2] MV group using KMA anesthesia. 3] MV group using KMA with 0.25 MAC isoflurane. 4] MV group using KMA with 0.75 MAC isoflurane. Cytokine levels were measured in lung homogenate and plasma. Leukocytes were counted in lung tissue.

Results:

Lung homogenates: MV increased pro-inflammatory cytokines in lung homogenate. In mice receiving KMA + isoflurane 0.75 MAC no significant increase in IL-1β was found compared with non-ventilated control mice.

Plasma: MV induced a systemic pro-inflammatory response. In mice anesthetized receiving KMA + isoflurane (both 0.25 MAC and 0.75 MAC) no significant increase in TNF-α was found compared with non-ventilated control mice.

Conclusions:

The present study is the first to show that isoflurane attenuates the pulmonary IL-1β and systemic TNF-α response following mechanical ventilation in healthy mice.

INTRODUCTION

Mechanical ventilation (MV) activates the innate immune system resulting in the release of cytokines,[1] which may cause lung injury.[2] This has been called Ventilator-Induced Lung Injury (VILI) and can develop into Acute Respiratory Distress Syndrome (ARDS), with a mortality rate in humans exceeding 40%.[3] Ventilator settings play an important role since using a lung protective ventilation strategy (low tidal volumes) is associated with lower incidence of ARDS.[4;5] The pathophysiology of VILI is poorly understood, but a role for pro-inflammatory pathways has been proposed.[6]

Previously, VILI has been studied in rodents using MV with tidal volume well beyond used in clinical practice (i.e>25 ml/kg).[7;8] In addition, most studies were performed in animals with pre-injured lungs,[9-11] limiting the clinical relevance of these models. Recently, data from our group showed that MV in healthy mice, using low tidal volumes (8 ml/kg) induces transient pulmonary and systemic inflammation.[12] In addition, Wolthuis et al.[13] showed that low tidal volume MV (6 ml/kg) increases plasma concentration of IL-6 and IL-8 in humans. Therefore, even protective MV induces a systemic inflammatory response in both mice and humans. As inflammation is associated with lung injury,[4] and peripheral organ dysfunction,[14] strategies to reduce MV-induced inflammation may appear of clinical relevance.

Isoflurane is widely used in general anesthesia and has profound protective immunological effects on the heart,[15] the brain,[16] and the kidneys.[17] Surprisingly, the effects of isoflurane on MV-induced pulmonary and systemic inflammation in healthy lungs has not been studied. Therefore, we hypothesized that isoflurane reduces MV-induced pulmonary and systemic inflammation. In order to test this hypothesis healthy mice were mechanically ventilated for 4 hours using a protective ventilation strategy.

METHODS

All experiments were approved by the Regional Animal Ethics Committee in Nijmegen and performed under the guidelines of the Dutch Council for Animal Care and The National Institutes of Health.

Animals:

Experiments were carried out in male C57BL6 mice (n=34) (Charles River, Sulzfeld, Germany) age 10 - 12 weeks, with a weight ranging from 23 to 28g.

Mechanical ventilation:

Anesthesia was induced and maintained with an intraperitoneal injection of a combination of ketamine, medetomidine and atropine (KMA-mix) as described previously.[12] Mice were orally

intubated and ventilated. In short, tidal volume was set at 8 ml/kg, frequency at 150 / min, PEEP at 4 cm H_2O and the FiO_2 at 0.4.[12] Throughout the experiment rectal temperature was monitored and maintained between 36°C and 37.5°C using a heating pad. In addition to the KMA-mix, assigned animals received an inspiratory isoflurane concentration of respectively 0.3% and 1.0% which corresponds with MAC values of 0.25 and 0.75.[18]

Study groups:

Animals were randomly divided into 4 groups (n = 6 per group): 1] Control (C) (n=6): after induction of anesthesia, these mice were immediately sacrificed without being ventilated. 2] MV (V): animals were ventilated for 4 hours using KMA anesthesia. 3] MV with low dose isoflurane (V-$I_{0.25}$): animals were ventilated for 4 hours using KMA anesthesia and 0.25 MAC isoflurane and 4] MV with high dose isoflurane (V-$I_{0.75}$): animals were ventilated for 4 hours using KMA and isoflurane 0.75 MAC anesthesia.

To assess the effect of 0.75 MAC isoflurane in addition to KMA on hemodynamic performance, pilot experiments were conducted. Two groups of mice (n = 5 per group) were mechanically ventilated in the presence of an intra-arterial catheter: I] four hours MV using KMA anesthesia, II] four hours of MV using KMA and isoflurane 0.75 MAC anesthesia. Continuous intraarterial carotid blood pressure was measured and arterial blood gas analysis was performed as described previously.[12] We decided not to include these animals for cytokine or histopathologic analysis to avoid possible cytokine response resulting from instrumentation induced tissue damage. Furthermore as instrumentation may result in some blood loss and a considerable amount of blood was needed for blood gas analysis, the availability of blood for cytokine measurements would be insufficient.

Preparation and analysis of lung tissue:

After the animals were sacrificed, blood was collected by exsanguination, centrifuged at 14000 rpm (13,000 g) (Eppendorf 5415 C, Nethler-Hinz GmbH, Hamburg) for 2 minutes and plasma was stored at -80°C. Immediately after exsanguination, heart and lungs were carefully removed *en block* via midline sternotomy. The right middle lobe was fixed for light microscopy. The remaining lung tissue was homogenized for determination of cytokine concentrations.

Levels of tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-10, and keratinocyte-derived chemokine (KC) in lung homogenate and plasma were measured using Enzyme-Linked Immunosorbent Assay (for TNF-α, IL-6 and IL10: CytoSet, BioSource, Camarillo California USA; for KC: ELISA-Kit, R&D systems, Minneapolis, Minnesota USA). Levels of IL-1α and IL-1β in lung homogenate and plasma were assessed using specific radio-immuno-assays developed in our laboratory.[19] Lower detection limits: IL-1α and IL-1β; 40 pg/ml, TNF-α; 32 pg/ml, IL-6; 160 pg/ml, IL-10; 16 pg/ml and KC; 160 pg/ml.

For light microscopy the material was fixed in 4% buffered formalin solution overnight at room temperature, dehydrated and embedded in paraplast (Amstelstad, Amsterdam, The

Netherlands). Sections of 4 µm-thickness were used for further analysis. The enzyme activity of leukocytes was visualized by enzyme histochemistry using chloracetatesterase staining (a histochemical stain for granulocytes). The evaluating pathologist was blinded for the group to which the animal had been assigned.

Statistical Analysis:

Data are expressed as means (SEM) when distributed normally (mean arterial pressure, leukocyte counts) and expressed as median (range) otherwise (cytokine concentrations). Statistical analysis was performed with SAS (SAS Institute Inc. Cary NC, USA) statistical procedures. Since cytokine concentrations are not normally distributed, Kruskal Wallis procedures were used, with post hoc comparisons of subgroups (Duncan). Data of a particular cytokine concentration variable were ranked, followed by ANOVA in the General Linear Models procedure using the MEANS procedure with the Duncan option and Bonferroni correction for multiple comparisons. For analysis of leukocyte counts ANOVA was used on non-ranked data with post hoc comparison of group means. The level of significance was set at $P < 0.05$.

RESULTS

Cardiorespiratory parameters:

After initial reduction in mean arterial pressure (MAP), the animals exhibited stable hemodynamic parameters throughout the course of the experiment (figure 1). No significant differences were found between mice receiving KMA and mice receiving KMA and isoflurane 0.75 MAC.
Blood gas analysis showed normal pH, arterial carbon dioxide tension ($PaCO_2$) and arterial oxygen tension (PaO_2) levels with a small decrease in base excess. Blood gas analysis after 4 hours of MV was not significantly different between mice receiving KMA and mice receiving KMA and isoflurane 0.75 MAC (table 1).

Cytokine release induced by mechanical ventilation:

LUNG HOMOGENATES: In KMA anesthetized mice, MV induced a pulmonary pro-inflammatory response as indicated by elevated concentration of IL-1α, IL-1β,TNF-α and IL-6 compared with unventilated control mice. MV did not increase levels of IL-1β in mice receiving KMA + isoflurane 0.75 MAC compared with unventilated control mice. Isoflurane did not affect the response of other measured cytokines (Figure 2).
PLASMA: In KMA anesthetized mice, MV induced a systemic pro-inflammatory response as indicated by elevated concentration of KC, IL-6 and TNF-α compared with unventilated control mice (figure 3). MV did not increase levels of TNF-α in mice receiving KMA + isoflurane (both 0.25 MAC and 0.75 MAC) compared with unventilated control mice (figure 3). Isoflurane did not

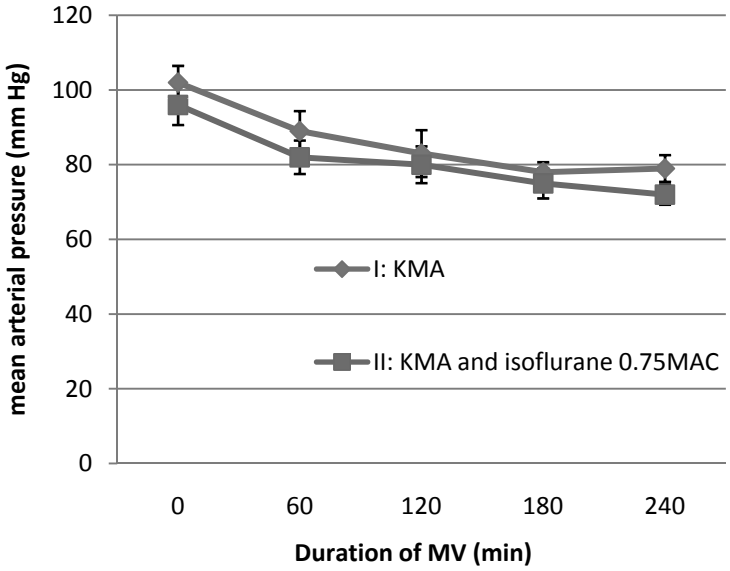

Figure 1. Mean arterial pressure during mechanical ventilation.
The animals with an intra-arterial cannula exhibited stable hemodynamic parameters throughout the experiments. Mean arterial pressure was within normal limits and remained above 65 mmHg in all animals. No differences were found between mice receiving KMA and mice receiving KMA with isoflurane 0.75 MAC. Data are expressed as mean (SEM).

Table 1. Arterial blood gas analysis after 4 hours of mechanical ventilation in mice receiving KMA and KMA with 0.75 MAC isoflurane.

	Blood gas values				
	pH	PaO_2 (mmHg)	$PaCO_2$ (mmHg)	HCO_3^- (mmol/l)	BE
I: KMA	7.35 (0.03)	194 (22)	36 (4)	19.6 (1.4)	-6.3 (0.9)
II: KMA and isoflurane 0.75 MAC	7.38 (0.02)	188 (16)	32 (2)	19.9 (0.5)	-5.7 (0.7)

Arterial blood gas analysis was only performed after 4 hours of mechanical ventilation. No samples were taken during mechanical ventilation, since this would result in hemodynamic instability due to excessive blood loss. No statistically significant differences were found between KMA and KMA with isoflurane 0.75 MAC. Values are mean (SEM). BE = base excess. I = 4 hours MV using KMA anesthesia, II = 4 hours of MV using KMA and isoflurane 0.75 MAC anesthesia.

affect the response of other measured cytokines. IL-1α and IL-1β remained below detection limit for all 4 groups (data not shown).

Histological examination:

MV was associated with significant elevation in the number of pulmonary leucocytes. Addition of isoflurane did not affect the number of pulmonary leukocytes following 4 hours of MV (table 2).

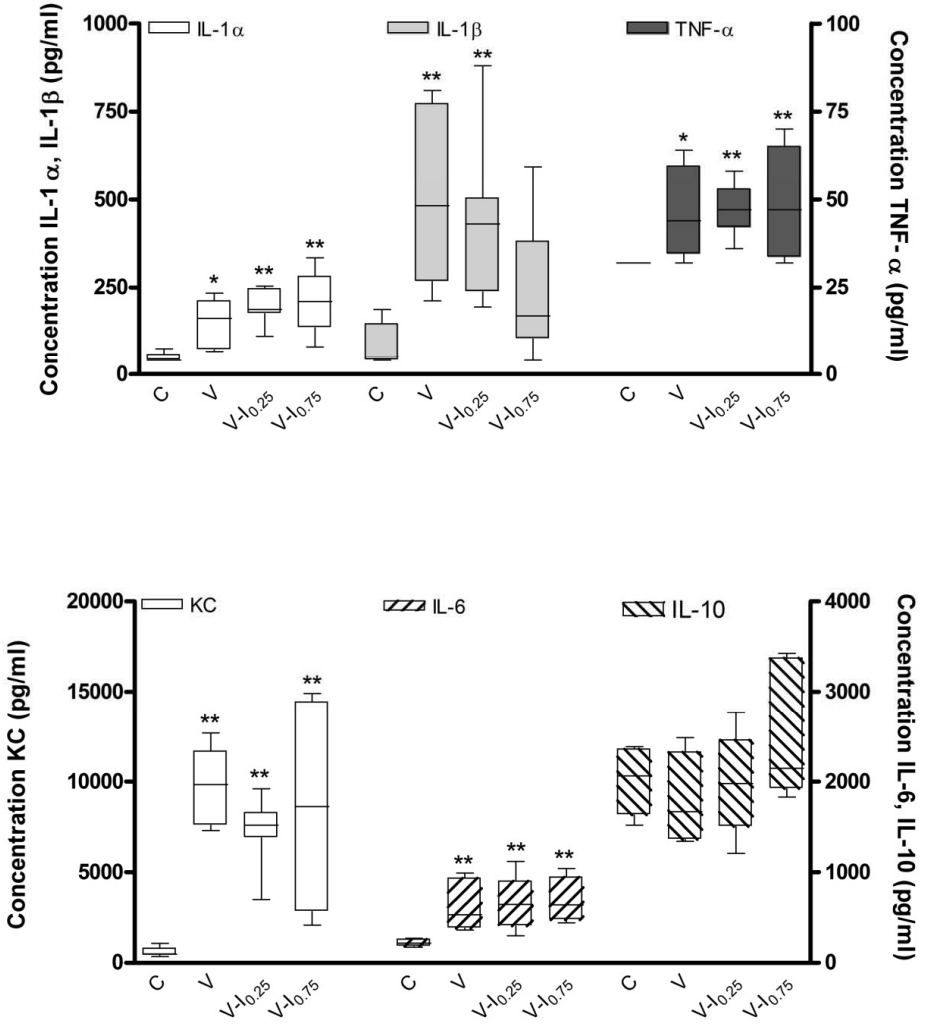

Figure 2. Cytokine levels in lung homogenates.
Mechanical ventilation significantly increased levels of interleukin (IL)-1α, IL-1β, IL-6, tumor necrosis factor-α and keratinocyte-derived chemokine in lung tissue homogenate when compared with unventilated animals (Group C). In mice receiving KMA + isoflurane 0.75 MAC mechanical ventilation did not increase IL-1β levels in lung homogenate compared with unventilated animals (Group C). Neither low-dose nor high-dose isoflurane did affect the response of other pro-inflammatory cytokines. Data are expressed as box (median, 25th and 75th percentiles) and whiskers (range). * $P< 0.05$ compared with unventilated animals (Group C); ** $P<0.01$ compared with unventilated animals (Group C). C = control group (unventilated mice); V = mice ventilated for 4 hours receiving KMA; V-$I_{0.25}$ = mice ventilated for 4 hours receiving KMA with 0.25 MAC Isoflurane; V-$I_{0.75}$ = mice ventilated for 4 hours receiving KMA with 0.75 MAC Isoflurane. - = lower detection limit.

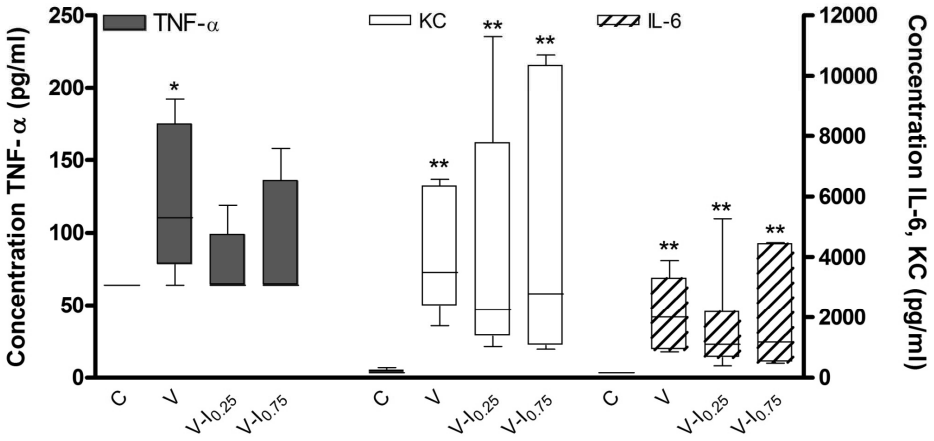

Figure 3. Cytokine levels in plasma.
Mechanical ventilation significantly increased levels of keratinocyte-derived chemokine (KC), interleukin (IL)-6 and TNF-α in plasma when compared with unventilated animals (group C). In mice receiving KMA with isoflurane (both 0.25 MAC and 0.75 MAC) mechanical ventilation did not increase TNF-α levels in plasma compared with unventilated animals (Group C). Data are expressed as box (median, 25th and 75th percentiles) and whiskers (range). * $P< 0.05$ compared with unventilated animals (Group C); ** $P<0.01$ compared with unventilated animals (Group C). C = control group (unventilated mice); V = mice ventilated for 4 hours receiving KMA; V-$I_{0.25}$ = mice ventilated for 4 hours receiving KMA with 0.25 MAC Isoflurane; V-$I_{0.75}$ = mice ventilated for 4 hours receiving KMA with 0.75 MAC Isoflurane. - = lower detection limit.

Table 2. Pulmonary leukocyte counts

Groups	Leukocytes x 10^{-4} / μm^2 Mean (SD)	p-value
Control	3.2 (1.0)	
Group V	6.1 (1.6)	< 0.0001
Group V-$I_{0.25}$	6.4 (1.7)	< 0.0001
Group V-$I_{0.75}$	7.6 (2.6)	< 0.0001

Values are mean (SEM)
MV = mechanical ventilation
p-values compared with Control group (unventilated animals)
Between the ventilated groups no significant differences were found.

DISCUSSION

The present study is the first to show that isoflurane attenuates the pulmonary IL-1β and systemic TNF-α response induced by MV in healthy mice. These findings may be of clinical importance as inflammation is proposed to play a role in VILI and organ dysfunction in mechanically ventilated patients.[4;14]

Cytokine release induced by mechanical ventilation:

In line with previous work from our group,[12] the present study confirms the pulmonary and systemic inflammatory response following 4 hours of low tidal volume MV in healthy lungs. Unlike inflammation following high tidal volume MV,[5;20] little has been published about the effects of low tidal volume MV on pulmonary and systemic inflammation. Caruso et al.[21] found an increase of IL-1β mRNA expression in lung tissue following low tidal volume MV. The present study extended this observation by showing increased IL-1β protein levels in lung homogenates following low tidal volume MV. Recently, it has been shown that pulmonary inflammation was significantly lower when using a protective ventilatory strategy (low tidal volume, PEEP 10 cmH_2O) compared with a non protective strategy (high tidal volume with zero PEEP) after 5 hours of MV in healthy lungs.[13] Nevertheless, in that study and in line with our observations low tidal volume MV did increase levels of pro-inflammatory cytokines in broncho-alveolar lavage (BAL) fluid and plasma.[13] In apparent contrast to our results are the findings of Dhanireddy et al.[22] who did not find an increase of KC in lung homogenates and plasma following low tidal volume MV using isoflurane 4% and Takenaka et al.[23] who also found no IL-6 and TNF-α increase in plasma following low tidal volume MV. However in both studies a trend towards an increase in pro-inflammatory cytokines could be observed. Accordingly, the present study confirms pulmonary and systemic cytokine increase following low tidal volume MV of healthy lungs.

Effect of isoflurane on inflammatory response:

Isoflurane attenuated the increase of pulmonary IL-1β and systemic TNF-α levels following 4 hours of low tidal volume MV. Previously, isoflurane was found to inhibit IL-1β induced cytokine production of alveolar epithelial type II cells,[24] which are widely exposed to isoflurane during general anesthesia.[25] In injurious models, isoflurane is also found to reduce endotoxin induced lung injury,[26] in which IL-1β and TNF-α are suggested to play an important role.[27] In addition, isoflurane attenuated peritoneal fluid IL-1β response in zymosan-induced peritonitis.[28] Furthermore isoflurane is found to reduce IL-1β in BAL fluid and IL-1β and TNF-α in plasma following endotoxemia.[29]

In apparent contrast to our findings, Kotani et al.[30] found isoflurane to increase gene expression of IL-1β and TNF-α in BAL fluid following MV of healthy rats. Despite this increase in gene expression, no significant increase in cytokine concentrations in lung lavage fluid was reported.[30]

In VILI various pro-inflammatory cytokines play a role including IL-1β and TNF-α. IL-1β has been shown to play a central role in lung injury following hemorrhage,[31] or liver injury.[32] as an early initiator of the inflammatory response. TNF-α is involved in pulmonary inflammation induced by high stretch MV in healthy lungs.[7] Both IL-1β and TNF-α are involved in the inflammatory response to low tidal volume MV in healthy lungs.[12;33] In the present study isoflurane prevented the increase of pulmonary IL-1β and systemic TNF-α levels following 4 hours of low tidal volume MV, therefore isoflurane may be of clinical importance in reducing VILI.

Methodological considerations:

The number of mice per group was chosen arbitrarily, based on our previous studies using this animal model.[12;34] As the effect of isoflurane on mechanical ventilation-induced inflammation was unknown, we could not perform adequate sample size analysis. Therefore, data obtained from the present study should be interpreted with caution.

Factors affecting cytokine response other than MV were carefully avoided. Experiments were performed in non-invasively monitored animals, after having documented cardiorespiratory stability in invasively monitored animals to eliminate the possibility of triggering an inflammatory response by insertion of an intra-arterial line.[33] In the current study mean arterial pressure was maintained above 65 mmHg and blood gas analysis showed normal pH, $PaCO_2$ and PaO_2 levels. Only a small decrease in base excess after 4 hours of MV was observed, comparable with other studies.[7;35] This slight decrease in base excess in the presence of a normal mean arterial pressure unlikely interferes with our observations.

Ideally a subgroup receiving only isoflurane (without ketamine) and a subgroup with spontaneously breathing animals receiving KMA-anesthesia should be included. However, the latter subgroup will result in hypoventilation with severe respiratory acidosis and hemodynamic instability, and since ketamine is known to have an inhibitory effect on cytokine production,[36;37] this will make comparison with a subgroup receiving only isoflurane inappropriate.

It is now commonly accepted that MV as well as volatile anesthetics have profound immunological effects.[38;39] Even low tidal volume MV leads to a pulmonary and systemic inflammatory response.[12;33] In this respect isoflurane may play a role.

In conclusion, isoflurane attenuates the pulmonary IL-1β and systemic TNF-α increase following 4 hours of low tidal volume MV in healthy mice.

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Chapter 7

General discussion

GENERAL DISCUSSION

Mechanical ventilation is a lifesaving intervention in the care of the critically ill patient. It is also used in millions of patients each week around the world to facilitate surgical interventions under general anesthesia. In the last 30 years numerous studies have provided evidence that lung injury is often associated with mechanical ventilation.[1-3] This Ventilator Induced Lung Injury (VILI) may impair the function of healthy lungs,[4] or exacerbate injury in the diseased lung (i.e. due to infection, trauma).[5]

Clinical studies have shown that the inflammatory response in the lung depends on ventilator settings. In patients with Acute Respiratory Distress Syndrome (ARDS) a "lung-protective" ventilation strategy attenuated inflammatory mediators,[6] and decreased mortality.[5] In these patients with severely injured lungs, it is extremely difficult to determine the specific contribution of mechanical ventilation to lung injury. In other words, whether mechanical ventilation per se induces pulmonary and systemic inflammation remains unknown. For a better understanding of the relevant pathophysiologic mechanisms underlying the contribution of mechanical ventilation to the development of VILI, we developed a mouse model for prolonged mechanical ventilation, with ventilator settings similar to those used in clinical practice.

In **chapter 2** it is shown that in our mouse model "noninjurious" or "lung-protective" ventilation does not exist and that even this protective ventilation can lead to an inflammatory response in healthy lungs. Fortunately, mechanical ventilation in elective, healthy patients rarely leads to clinical relevant injury. Apparently, the trigger induced by the ventilator is relatively mild and the lung is able to cope with the inflammatory response. The inflammatory changes observed in healthy lungs may just be physiologic adaptations to the artificial process of mechanical ventilation. However, the inflammatory response following mechanical ventilation may be clinically relevant as this forms the basis for the "two-hit hypothesis". This proposes that injury (*e.g.*, the critically ill patient) primes the immune system ("first hit") for a lethal inflammatory reaction to a later, otherwise (usually) nonlethal, secondary insult (second hit), namely mechanical ventilation.[7-9] This can result in full-blown ARDS with its high morbidity and mortality.[5;10;11]

Increasing evidence in rodents and in humans further support our findings of mechanical ventilation-induced inflammation in the absence of a priming pulmonary insult, even when applying currently considered "optimal" (least injurious) ventilator settings.[12-14]

The mechanisms underlying the inflammatory response following mechanical ventilation are incompletely understood. A role for pro-inflammatory pathways has been proposed.[1] Toll-like receptors (TLRs) are essential in host defense by starting an inflammatory response after recognizing pathogens in infectious lung injury.[15] However recent studies indicate that TLRs (especially TLR4) also plays a role in non-infectious lung injury.[16-19] In **chapter 3** it is confirmed that in our mouse model TLR4 plays an important role in the inflammatory response induced

by mechanical ventilation in healthy lungs. Most likely, endogenous ligands for TLR4 initiate this process. Previously, different endogenous ligands for TLR4 have been identified including heat shock protein 60,[20] fibronectin,[21] heparan sulfate proteoglycan,[22] biglycan,[23] hyaluronan[24] and the myeloid-related proteins 8 and 14.[25] Which endogenous ligands are responsible for activating TLR4 in the inflammatory response following mechanical ventilation in healthy lungs remains to be investigated.

To attenuate the inflammation induced by mechanical ventilation, TLR4 may be a useful therapeutic target. However, since TLR4 was first identified as the key identifier of lipopolysaccharide, blocking this receptor will increase sensitivity to infection.[26] We therefore investigated the pathway further downstream of TLR4, which is complex. TLR4 activation may stimulate two distinct post receptor pathways. First, a MyD88 pathway resulting in rapid translocation of nuclear factor (NF)-κB into the nucleus, and transcription of proinflammatory genes inducing cytokine production.[15;27] Second, a TRIF (Toll/interleukin-1 receptor domain-containing adapter inducing interferon-β) dependent pathway resulting in delayed NF-κB translocation. This pathway is also associated with enhanced cytokine production.[27-29] Surprisingly, the TRIF pathway was recently found to mediate injury in the absence of an infectious agent.[30] Given the fact that the MyD88 pathway is critical to the response to bacterial infections, new strategies to modulate the TRIF pathway while leaving the MyD88 pathway intact, might be beneficial in some forms of lung injury.[27] Accordingly, we investigated TRIF involvement in our model of VILI. In **chapter 4** it is shown that TRIF deletion prevented the increase in plasma IL-6 and attenuated pulmonary IL-1β levels following mechanical ventilation, by reducing NF-κB activation. IL-1β has been shown to be among the most biologically active cytokines in the lungs,[31;32] and Il-6 is associated with the development of distal organ failure in VILI.[5;33] Therefore it is likely that in downstream signaling of TLR4, the TRIF pathway is involved and may be an effective strategy to prevent or reduce mechanical ventilation-induced pulmonary and systemic inflammation.

As mechanical ventilation-induced inflammation is associated with lung injury,[5] and peripheral organ dysfunction,[34] and persistent cytokine elevation is associated with a poor outcome in patients with ARDS,[7;8] strategies to reduce mechanical ventilation-induced inflammation might be of clinical relevance. Low tidal volume mechanical ventilation was designed to protect the lungs from excessive stretch. Clinical trials indeed showed that ventilator management, by limiting tidal volume and by maintaining recruitment of alveolar regions with sufficient PEEP, can alter mortality in patients with ARDS.[5;3515] These "lung-protective" mechanical ventilation strategies reduced but could not prevent VILI.[4] However using low tidal volumes may result in an increase in $PaCO_2$ ("permissive hypercapnia"). One can wonder if acceptance of hypercapnia with lung-protective ventilation should lead us to intentionally use hypercapnia in patients with acute lung injury. Several studies support this hypothesis. Hypercapnia has been shown

to reduce ventilator-induced lung injury,[36;37] and to be remarkably well tolerated.[38] It may even improve outcome in ARDS patients,[38;39] possibly by attenuating inflammation.[40] We therefore investigated the effects of hypercapnia on the inflammatory response following mechanical ventilation. In **chapter 5** we demonstrate that hypercapnia attenuates mechanical ventilation-induced inflammation. Following mechanical ventilation, hypercapnic acidosis by itself directly attenuated pulmonary leukocyte influx and cytokine release during mechanical ventilation while all other cardiorespiratory and ventilatory parameters remained identical between ventilated groups. Therefore, hypercapnic acidosis may represent an important pathway to protect the lung against VILI.

Isoflurane is widely used in general anesthesia and has profound protective immunological effects on the heart,[41] the brain,[42] and the kidneys.[43] Studies of the pulmonary effects of isoflurane are limited to injurious models, in which isoflurane is found to reduce inflammation in endotoxin induced lung injury.[44;45] Whether isoflurane can attenuate the inflammatory response in the healthy lung following mechanical ventilation is unknown. In **chapter 6** it is shown that isoflurane indeed attenuates this inflammatory response induced by mechanical ventilation in healthy mice. Pulmonary IL-1β and systemic TNF-α levels during mechanical ventilation were lower in the presence of inhaled isoflurane. Both IL-1β and TNF-α are important in the inflammatory response to low tidal volume mechanical ventilation in healthy lungs. Therefore, isoflurane may be of clinical importance in reducing VILI.

Future directions:

Studies in this thesis show that even a so-called protective mode of ventilation leads to an inflammatory response in healthy lungs. Fortunately, this inflammatory response is relatively mild and mostly of little clinical significance. Nevertheless, we investigated the effects of mechanical ventilation in healthy lungs to shed more light on the pathophysiological mechanisms involved. The knowledge gained may help us understand its role in the two-hit scenario. It is clear that, from a clinical point of view, the next step should be to investigate this two-hit scenario, which more closely resembles the clinical situation in which critically ill patients are mechanically ventilated. Therefore in the future a 'second hit' model needs to be developed.

From a purely scientific point of view, human studies are needed to confirm the effects we found in mechanically ventilated healthy mice. From an ethical point of view it is difficult to mechanically ventilate healthy humans just for scientific reasons. Previously performed human studies have investigated mechanical ventilation in patients who underwent surgery. However, it is clear that in these studies the inflammatory effects of the surgical procedure cannot be excluded.

To gain more insight in the pathophysiological mechanisms involved in mechanical ventilation, several questions need to be addressed. First, a search "upstream" to identify the specific endogenous ligands which activate TLR4. Second, more research is needed to analyze the specific intracellular factors involved. Previously, TNF receptor associated factor 6 (TRAF 6) was found to mediate the delayed NF-κB activation initiated by TRIF.[27;46] However, what other factors are involved and specifically, what key molecular "switch" controls activation of MyD88 or TRIF remains unclear. Besides factors initiating the inflammatory response little is known about stimuli that maintain the inflammatory response during prolonged mechanical ventilation.

In this thesis the influence of hypercapnia and the use of isoflurane were investigated.
From our experiments it remains unclear whether it is the hypercapnia or the resultant acidosis that is responsible for the observed beneficial effects. This needs further investigation.
Since isoflurane is so widely used in general anesthesia its protective pulmonary immunological effects need to be studied in humans. More research is clearly needed to identify new strategies in reducing the negative side effects of mechanical ventilation.

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Chapter 8

Summary

SUMMARY

This thesis describes the effects of mechanical ventilation in healthy lungs. We developed an animal model suitable to study the inflammatory response following mechanical ventilation. Using this model, we investigated the pathophysiological mechanisms underlying Ventilator Induced Lung Injury (VILI) by analyzing the role of Toll-like receptor (TLR) signaling. We also examined possibilities of modifying the inflammatory response following mechanical ventilation in the healthy lung.

Chapter 1 reviews the literature concerning VILI. Mechanical ventilation is an indispensable tool in the management of critically ill patients and during surgery under general anesthesia. Despite the life saving effect, mechanical ventilation can also exacerbate or perpetuate lung injury. Different theories on the underlying mechanism of VILI, clinical aspects of VILI and the contribution of different modes of mechanical ventilation are discussed. Higher levels of cytokines are associated with poorer clinical outcome. Stretching of pulmonary and non-pulmonary cells by mechanical ventilation increases synthesis of cytokines, a process called mechanotransduction. Toll-like receptors (TLRs) form the core of our innate immune system by recognizing molecules that are broadly shared by pathogens. Recently, it was discovered that TLRs not only recognize exogenous microbial products, but also endogenous ligands. In the development of non-infectious lung injury TLR2 and TLR4 play an important role. The involvement of cytokines and the different TLRs in VILI are discussed. Finally, possibilities of modulating VILI are addressed. "Lung-protective" mechanical ventilation protects the lung from excessive stretch, which reduces but does not prevent VILI. Permissive hypercapnia and the use of isoflurane are described as possibilities of modulating VILI.

Chapter 2 describes the effects of mechanical ventilation in the healthy lung, using ventilatory protocols similar to those currently used clinically during general anesthesia and in the intensive care unit. Mechanical ventilation in healthy mice induces a reversible inflammatory reaction, while preserving tissue integrity. This model shows that "noninjurious" or "lung-protective" ventilation may not exist and that even this careful mode of ventilation leads to a reversible inflammatory response. Furthermore this model offers opportunities to study the pathophysiological mechanisms of VILI and the contribution of mechanical ventilation to the "multiple-hit" concept.

Chapter 3 analyses the involvement of TLR2 and TLR4 receptor signaling in the inflammatory response induced by mechanical ventilation in the healthy lung. The results show that TLR2 signaling does not play a role in the inflammatory response following mechanical ventilation in healthy mice. Instead, several key findings regarding the role of TLR4 signaling are revealed. Mechanical ventilation resulted in elevated expression of endogenous TLR4 ligands

in broncho-alveolar lavage fluid and enhanced mRNA levels for TLR4 in lung homogenates. The increase of inflammatory cytokines in the lung and the systemic inflammatory response in plasma following mechanical ventilation appeared to be at least partially TLR4 dependent, supporting a role for TLR4 in the inflammatory reaction following mechanical ventilation in healthy lungs. These are important findings, as they may provide guidance for therapeutic strategies.

Chapter 4 analyses the involvement of Toll/interleukin-1 receptor domain-containing adapter inducing interferon-β (TRIF), a protein downstream of TLR4. Activation of TRIF causes delayed translocation of nuclear factor (NF)-κB into the nucleus, and transcription of proinflammatory genes inducing cytokine production. The increase of inflammatory cytokines in the lung and the systemic inflammatory response in plasma following mechanical ventilation appeared at least partially TRIF dependent, since it was found that TRIF deficiency attenuated inflammation.

Chapter 5 analyzes the effect of hypercapnic acidosis on mechanical ventilation-induced inflammatory response. A series of experiments is described to establish the effect of hypercapnic acidosis on pulmonary cytokine increase and leukocyte influx by administration of inhaled CO_2, while maintaining identical ventilator settings. Hypercapnic acidosis significantly decreases pulmonary leukocyte influx and cytokine release following mechanical ventilation.

Chapter 6 analyzes the effect of isoflurane on mechanical ventilation-induced inflammatory response. A series of experiments were performed in which mice were ventilated receiving different inspiratory concentrations of isoflurane to establish its effect on pulmonary cytokine increase and leukocyte influx following mechanical ventilation. Isoflurane attenuates the pulmonary IL-1β and systemic TNF-α response induced by mechanical ventilation in healthy mice.

Chapter 9

Samenvatting

SAMENVATTING

Dit proefschrift beschrijft de relatie tussen beademing en het ontstaan van een ontstekingsreactie. Wij ontwikkelden een diermodel waarmee aangetoond werd dat kunstmatige beademing van gezonde longen een ontstekingsreactie genereert in de long zelf, maar ook in andere organen. Dit model maakt het mogelijk om onderzoek te doen naar onderliggende pathofysiologische mechanismen van beademings-geïnduceerde longschade en de rol van de Toll receptoren 2 en 4 daarbij. Tevens hebben we met dit model onderzoek gedaan naar mogelijkheden om deze ontstekingsreactie als gevolg van beademing te remmen.

Kunstmatige beademing is levensreddend bij de behandeling van kritisch zieke patiënten met respiratoir falen en onmisbaar tijdens operaties onder algehele anesthesie. Echter, kunstmatige beademing kan reeds bestaande longschade verergeren of nieuwe longschade doen ontstaan. Ondanks het levensreddende effect heeft kunstmatige beademing dus ook ongewenste bijeffecten. In **hoofdstuk 1** wordt de bestaande literatuur aangaande beademings-geïnduceerde longschade samengevat. De verschillende theorieën over de onderliggende mechanismen van beademings-geïnduceerde longschade worden beschreven evenals de klinische aspecten, waarbij ingegaan wordt op de bijdrage van de verschillende vormen van kunstmatige beademing.

Kunstmatige beademing stimuleert de aanmaak van ontstekingseiwitten als gevolg van rek op de long. Dit proces wordt mechanotransductie genoemd. Hogere concentraties van ontstekingseiwitten zijn geassocieerd met een slechtere prognose. De betrokkenheid van ontstekingseiwitten (onder andere cytokines en witte bloedlichaampjes) bij het ontstaan van beademings-geïnduceerde longschade wordt besproken.

Toll receptoren vormen de basis van onze eerste afweer tegen ziekteverwekkers van buiten. Recent heeft men ontdekt dat deze receptoren niet alleen schadelijke lichaamsvreemde stoffen, maar ook lichaamseigen stoffen, zogenaamde endogene liganden kunnen herkennen. De betrokkenheid van Toll receptoren bij het ontstaan van longschade wordt besproken evenals het werkingsmechanisme van deze Toll receptoren daarbij.

Tot slot wordt een aantal mogelijkheden om beademings-geïnduceerde longschade te remmen beschreven. Kunstmatige beademing met kleine volumina beschermt de long tegen overmatige rek, hetgeen de longschade vermindert maar niet voorkomt. Hypercapnie, oftewel het opzettelijk hoog houden van het koolstofdioxide gehalte in het bloed, wordt gezien als mogelijke remmer van beademings-geïnduceerde longschade. Isofluraan is een dampvormig anesthesticum dat over de hele wereld gebruikt wordt bij kunstmatige beademing onder algehele anesthesie. Het gebruik van isofluraan kan de ontwikkeling van beademingsgeïnduceerde longschade mogelijk remmen.

Hoofdstuk 2 beschrijft de gevolgen van kunstmatige beademing in de gezonde long bij muizen, waarbij gebruik wordt gemaakt van beademingsinstellingen die vergelijkbaar zijn met de instellingen die gebruikt worden om patiënten op een operatiekamer of intensive care te beademen. Dit diermodel laat zien dat "niet schadelijke" of "longprotectieve" beademing niet bestaat en dat zelfs een voorzichtige manier van beademen leidt tot een ontstekingsreactie.

Hoofdstuk 3 analyseert de betrokkenheid van de Toll 2 en de Toll 4 receptor bij het ontstaan van de ontstekingsreactie als gevolg van kunstmatige beademing. De Toll 2 receptor blijkt geen rol te spelen bij het ontstaan van beademings-geïnduceerde longschade. Echter, een aantal zeer belangrijke bevindingen worden gedaan betreffende de Toll 4 receptor. Kunstmatige beademing resulteerde in een verhoogde expressie van endogene liganden voor deze receptor. Ook bleek de gevonden stijging van ontstekingseiwitten in de long en in het bloed afhankelijk te zijn van de aanwezigheid van de Toll 4 receptor.

Hoofdstuk 4 analyseert de betrokkenheid van TRIF (Toll/interleukin-1 receptor domain-containing adapter inducing interferon-β), een eiwit dat intracellulair als boodschapper fungeert na activatie van de Toll 4 receptor. De afwezigheid van TRIF verminderde de vorming van ontstekingseiwitten in de long en het bloed, hetgeen een belangrijke rol voor TRIF ondersteunt bij beademings-geïnduceerde longschade in gezonde longen.

Hoofdstuk 5 analyseert het effect van hypercapnie op beademings-geïnduceerde longschade in gezonde longen. Hypercapnie is een verhoogd koolstofdioxidegehalte in het bloed. Een reeks experimenten wordt beschreven om de effecten te onderzoeken van hypercapnie op de ontstekingseiwitten die ontstaan als gevolg van kunstmatige beademing. Hypercapnie blijkt een remmend effect te hebben op beademings-geïnduceerde longschade.

Hoofdstuk 6 analyseert het effect van isofluraan op beademings-geïnduceerde longschade. Isofluraan is een dampvormig anestheticum dat gebruikt wordt om iemand onder narcose te houden. Een reeks experimenten wordt beschreven om de effecten te onderzoeken van de inhalatie van isofluraan op de ontstekingseiwitten die ontstaan als gevolg van kunstmatige beademing. Isofluraan blijkt een remmend effect te hebben op beademings-geïnduceerde longschade.

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Michiel

CURRICULUM VITAE

Michiel Vaneker werd geboren op 27 augustus 1975 te Apeldoorn. Vanaf zijn tweede levensjaar was hij woonachtig in Vorden. In 1993 en 1995 behaalde hij respectievelijk het HAVO en het VWO diploma aan het Baudartius College in Zutphen. In verband met uitloting voor de studie geneeskunde werd aangevangen met de studie medische biologie aan de Vrije Universiteit te Amsterdam. Later dat jaar werd hij nageplaatst en kon alsnog gestart worden met de studie geneeskunde aan de Radboud Universiteit te Nijmegen. In 1999 en 2001 werd aan deze universiteit respectievelijk het doctoraal examen en het artsexamen (cum laude) afgelegd. Van 1990 tot en met 2001 was hij naast zijn opleiding werkzaam bij Hotel Bakker in Vorden.

In 2002 werd hij aangenomen voor de opleiding tot anesthesioloog bij de afdeling Anesthesiologie van het Universitair Medisch Centrum St Radboud te Nijmegen onder leiding van prof. dr. L.H.D.J. Booij. Tijdens de opleidingsperiode werd de interesse voor wetenschappelijk onderzoek gewekt en onder verantwoordelijkheid van prof. dr. G.J. Scheffer kwam hij terecht op het laboratorium experimentele anesthesiologie. In 2004 startte hij met het opzetten van de onderzoekslijn die uiteindelijk resulteerde in dit proefschrift. Een groot deel van dit proefschrift kwam dan ook tot stand tijdens de opleidingsperiode. Sinds september 2007 is hij werkzaam als anesthesioloog in het Universitair Medisch Centrum St Radboud te Nijmegen. Tevens maakt hij deel uit van het team van de Lifeliner 3, het mobiel medisch team van de Acute Zorg Regio Oost dat gestationeerd is op de vliegbasis Volkel.

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